UNIVERSITY OF HAWAII
LIBRARY
OCT 19 '51

EXPERIMENTAL CELL RESEARCH

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

EDITORS:

TORBJÖRN CASPERSSON · HONOR B. FELL · JOHN RUNNSTRÖM

FRANCIS O. SCHMITT · PAUL WEISS · RALPH W. G. WYCKOFF

VOLUME II · SEPTEMBER 1951 · NUMBER 3

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
Honor B. Fell, Strangeways Laboratory, Cambridge, England
J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden
Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Paul Weiss, University of Chicago, Chicago 37, Illinois
Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

EXECUTIVE COMMITTEE:

T. Caspersson, Honor B. Fell, J. Runnström and J. F. Danielli, King's College, London, W. C. 2, England.

ADVISORY BOARD:

H. Bauer (Wilhelmshafen), W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), A. Buzzati-Traverso (Verbania Pallanza), R. Chambers (New York), E. Chargaff (New York), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbor, N. Y.), J. Engelbreth-Holm (Copenhagen), E. Fauré-Frémiet (Paris), A. Fischer (Copenhagen), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa.), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holmgren (Stockholm), H. Holter (Copenhagen), J. Holtfreter (Rochester, N. Y.), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Bloomington, Ind.), A. Lwoff (Paris), A. Mirsky (New York), G. Montalenti (Naples), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York), J. Randall (London), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa.), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A. Tyler (Pasadena, Calif.), W. Wilbrandt (Bern).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in four issues annually at $12.00 per volume. The price of one issue is $3.50. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

THE MECHANICS OF BUDDING AND COPULATION IN SACCHAROMYCES[1]

CARL C. LINDEGREN

Southern Illinois University, Carbondale, Ill.

Received August 7, 1950

THE MECHANICS OF BUDDING AND COPULATION IN SACCHAROMYCES

Several conspicuous autonomous structures in the yeast cell are partitioned between mother and bud. The process by which chromosomes are equally divided has been described (3). In addition to the chromosomes, however, there are other permanent structures which never originate *de novo*. They have the same type of continuity in time as chromosomes but are less precisely partitioned than the latter. During growth these nonchromosomal entities may regain their normal functional proportions by virtue of the fact that they are rate-limiting. Those structures which are present in excess are delayed until the deficient structures accumulate in adequate amounts to maintain metabolic activity, or the cell dies. This argues that the nonchromosomal structures are relatively homogeneous and that a change in quantity could suffice to restore the previous equilibrium,—a mechanism which would not operate with the extremely non homogeneous chromosomes.

1. *The cytoplasm.* The cytoplasm is a limpid fluid which is transmitted to each daughter cell. It is rich in RNA but varies in basophily and contains the mitochondria, usually adhering to the surface of the centrosome or the nuclear vacuole. In addition, the cytoplasm occasionally contains deposits of glycogen which sometimes pervade the entire cytoplasm. The state of the mitochondria varies from highly refractile structures, sharply defined from the cytoplasm by a lipoidal layer, to less refractile organelles with somewhat irregular boundaries. The limpid cytoplasm is a permanent cell structure but the mitochondria appear to be formed *de novo*. At least, they are more abundant at some times than at others. I have suggested that the mitochondria function in establishing dormancy by insulating various cell components inside an oily layer and thus separating some essential ingredients from the reactive cytoplasm.

2. *The centrosome.* The centrosome is a solid and rigid structure which stains readily with acid fuchsin but does not stain with basic dyes. This highly basic

[1] This work has been supported by grants from Anheuser-Busch, Inc. and the United States Public Health Service.

organelle may contain some of the histones which Caspersson found in chromosomes. The centrosome is always attached to the nuclear vacuole and is the most rigid structure in the cell as revealed by its behavior following shrinkage of the cell. It never originates *de novo* and plays a leading part in budding, copulation and meiosis.

3. *The centrochromatin.* The centrochromatin is a basophilic, Feulgen-positive substance closely attached to the basic centrosome (probably by an acid-base reaction), with some portion of it usually in contact with the nuclear vacuole. It is partitioned between the cells following budding by a direct division controlled by two tiny centrioles. In the resting cell it may appear to assume a spherical form by covering most of the centrosome; in division it is usually present in the form of a long strand. The centrosome and centrochromatin have been identified with the nucleus by several workers but this view has been criticized by Lindegren (3), Lindegren and Rafalko (4) and Rafalko and Lindegren (5).

In agreement with other workers, I previously described the centrochromatin as composed of two parallel strands, or of numerous small particles. The apparent doubleness is an illusion arising from the fact that the filament often bends on itself to assume a V- or U-shape. The appearance of numerous small particles is due to poor fixation and is especially prevalent in preparations fixed with alkali. The view that the centrochromatin is a single filament external to the centrosome is supported by a multitude of observations on well fixed cells. Centrochromatin is probably homologous to the heterochromatin of higher forms differing only in being carried on the centrosome rather than the chromosome.

4. *The nuclear vacuole and its contents.* The nuclear vacuole contains the chromosomes and the nucleolus. The chromosomes are partitioned between mother and bud vacuole, in a precise orderly manner without recourse to a spindle. The wall of the nuclear vacuole does not break down at any time in the life cycle; it is a permanent cellular structure.

5. *The cell wall.* The cell wall may not be a permanent cell structure since it is formed *de novo* in the spores.

The behavior of the chromosomes in mitosis has been described (3) and our observations to date have generally confirmed this earlier description. The centrosome, however, plays a part in the process of budding considerably different from that originally described and the present paper supercedes the earlier description of the budding mechanism (2).

STAINING TECHNIQUE

Budding cells were fixed with Schaudinn's fluid and stained with Heidenhain's haemotoxylin. The mitochondria are apparently destroyed by the fixation and neither the chromosomes nor the nucleolus is visible after this procedure has been used probably because the cytoplasm contains such large amounts of RNA that the drastic destaining required to differentiate the other cellular organelles destains the chromosomes almost completely. This makes it easier to follow centrochromatin, centrosome, vacuole and cytoplasm. The yeast cell contains 4–7 per cent RNA (dry weight) and 0.1–0.3 per cent DNA (Ogur, personal communication) an unusually high ratio. More than three-quarters of the DNA appears by inspection to be in the

centrochromatin leaving only a very small amount of DNA in the slender filamentous strands which comprise the chromosomes. The difficulty encountered in staining yeast chromosomes by conventional methods arises from the remarkably small ratio of DNA to RNA in the cell. This is the basis for the incorrect assumption by many workers that the centrosome and centrochromatin comprise the nucleus.

The copulating cells were stained by covering a few drops of cells with about 1 ml of Barrett's hemotoxylin: 2 parts glacial acetic acid, 1 part 0.5 per cent hemotoxylin, 1 part 4 per cent iron alum. After a few hours a few drops of aceto-orcein were added. This stain is capricious but exceptionally effective when it works well. The cells are kept in the stain in corked tubes and examined in the stain. The chromosomes and centrosomes are visible and in some preparations the centrochromatin is very clear. The cell wall is sharply defined and the centrochromatin either is contained in a pellicle or has shrunk from the cytoplasm.

BUDDING

Figure 1 shows a cell in which the acidophilic centrosome attached to the nuclear vacuole is surrounded by the darkly staining cytoplasm. A band of basophilic centrochromatin is securely applied to the side of the centrosome and is also in contact with the nuclear vacuole. Greater differentiation often reveals a small centriole at each end of this band. The nuclear contents are unstained. The slight depression at the base of the cell into which the vacuole fits is the bud scar from the previous budding (1). This extension of the vacuole probably corresponds to the "cytoplasmic clear area" described by Lindegren and Rafalko (4).

Figure 2 shows the first step in the process of budding. The centrosome produces a small conical process which forces its way through the cytoplasm and erupts into the new bud shown in Fig. 3.

Figure 4. The nuclear vacuole sends out a long, slender process which follows the centrosome into the bud. Although the cell wall is not visible in these preparations it must be assumed that the cell wall never ruptures but is extended to enclose the bud at all times. The process from the nuclear vacuole appeared to be much less rigid than the centrosome. The vacuolar process follows the external surface of the centrosome into the bud, lying between the cell wall and the centrosome.

Figure 5 shows a cell in which the bud vacuole has received its two-stranded chromosome complex; this is an exception to the rule that the chromosomes are usually completely destained in the differentiation by iron alum.

In Figure 6 the bud vacuole is lobed; this is a rather common phenomenon. The cytoplasm has passed into the bud and completely surrounds the cent-

rosome and the bud vacuole. The extension of the centrochromatin along the surface of the acidophilic centrosome has begun.

Figures 7, 8 and 9 show cells in which the separation of the centrochromatin has been completed with mother and bud held together by the centrosome and the cell wall.

In Figure 10 the division of the centrosome is complete but both centrosomes are near the point of budding. In Figure 11 the bud centrosome has reached the distal end of the bud while the mother cell centrosome still lies in the neighborhood of the point of budding. In Figure 12 both centrosomes have reached the distal ends of the cells and are prepared for the formation of the next bud.

THE BUD SCAR

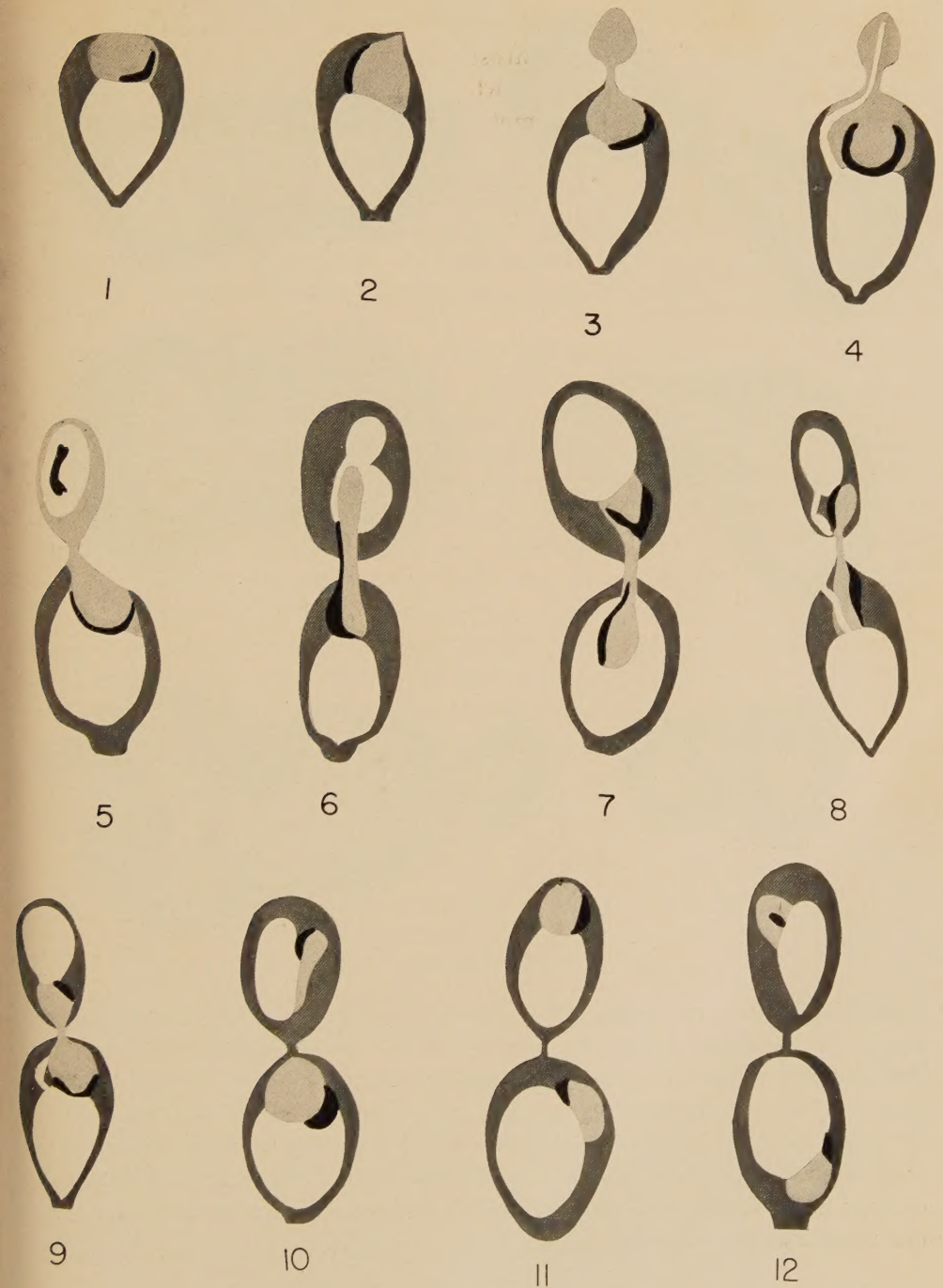
Barton described the bud scar as follows: "Each scar is seen to consist of a slightly raised circular rim (2 μ in diameter) enclosing a central thickened plug." The cell wall is not visible in these preparations but the "plug" of centrosomal material which holds bud and mother cell together is quite clear. The cell wall does not stain in these preparations and the "plug" passes through the cell wall, which must be projected in the imagination outside the darkly stained cytoplasm of each cell.

COPULATION

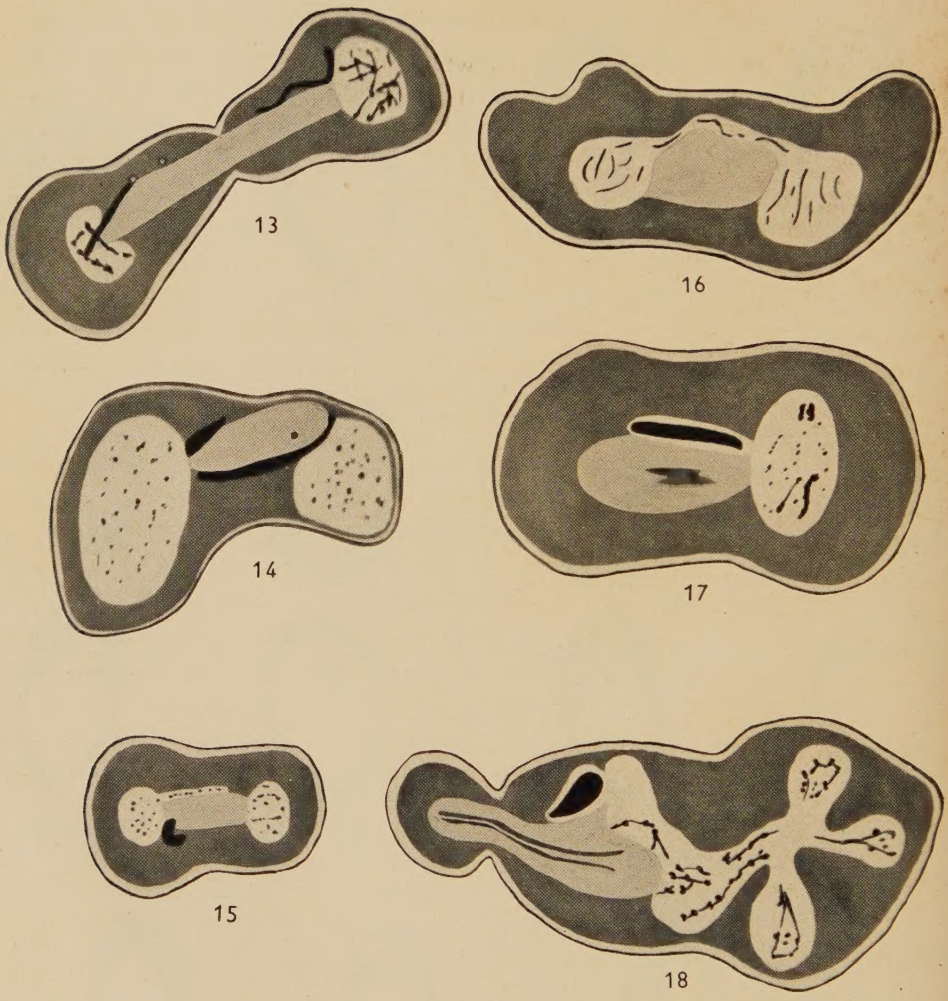
The process of copulation parallels the budding process in reverse. The copulation tube (like the young bud) contains only the centrosome, thus explaining Winge's rule that cells never copulate and bud at the same time. They must do either one or the other. Figure 13 shows the fusion of centrosomes. The fusion of cytoplasms follows (Figure 14). The bands of centrochromatin from each of the copulants travel along the centrosome to fuse into a single band, forming the centrochromatin of the new hybrid (Figures 15 and 17). Next one or both vacuoles send out processes which travel along the centrosome to fuse at their tips (Figures 15 and 16). After the fusion the contents of one vacuole flow through the fusion tube into the other. The zygote buds to produce a cell of the new diploid hybrid (Figure 18).

Genes and Other Self-Reproducing Organelles

One implication of the gene theory is that genes alone have the capacity for self-reproduction from an unlike environment. Observation of the permanent structures of the yeast cell shows that there are other autonomous



Figs. 1–12.



Figs. 13–18.

structures in the cell beside chromosomes and genes. The view that genes alone are self-reproducing has been carried to great lengths; some contend that autonomous extranuclear structures capable of self-reproduction are genic. The demonstration of several autonomous non genic structures in the yeast cell, which are essential in the sense that they reproduce themselves from their environment and that their failure to do so would automatically destroy the cell,' shows that capacity for self-reproduction is not an unique characteristic of the gene.

SUMMARY

The yeast cell contains a nucleus whose rigid centrosome carries a band of Feulgen-positive chromatin (centrochromatin) on its surface. The first step in budding is the formation of the bud by an extension of the centrosome over which the cell wall persists. Next the nuclear vacuole extends a process into the bud which contains the chromosomes. Finally the centrochromatin divides directly and the cells separate; a plug either of centrosome or cytoplasm sealing the bud pore. The cytoplasm, the centrosome, the centrochromatin and the nuclear wall are autonomous non genic organelles which never originate *de novo*.

Copulation is the reverse of budding. The centrosomes fuse first; the cytoplasms mix; the nuclear vacuoles fuse by processes which travel along the fused centrosomes; and finally the centrochromatins fuse to form a single band.

Figures 1–12. Drawings of budding yeast cells fixed in Schaudinn's fluid and stained with iron alum hemotoxylin, mounted in balsam. The cell wall is not visible due to the clearing action of the balsam. Except for Figure 5, the chromosomes and the nucleolus in the nuclear vacuole have been completely destained. The bud scar described by Barton is shown clearly at the end of the cell distal from the centrosome. The nuclear vacuole is usually forced into the extrusion formed by the bud scar. Since the cell wall is not visible, the plug of material connecting bud and mother cell as shown in Figure 12, fits into the cell wall and probably corresponds to the plug in the bud scar described by Barton. The details of the budding process are described in the text.

Figures 13–18. Copulating yeast cells stained with Barrett's hemotoxylin and aceto-orcein and mounted in the stain. Chromosomes are visible in the nuclear vacuoles. The centrosome is usually visible and often appears to have a core which stains differentially. Except in Figure 16, the centrochromatin is visible as darkly stained material; in some cases surrounded by a clear zone. The "thick waisted" form of the cells identifies them as derived from recent copulations and distinguishes them from budding cells. The process of copulation is discussed in the text.

REFERENCES

1. Barton, A. A., *J. Gen. Microbiol.*, **4**, 84 (1950).
2. Lindegren, Carl C., *Mycologia*, **37**, 767 (1945).
3. —— The Yeast Cell, its genetics and cytology. Educational Publishers Inc., St. Louis, Miss., 1949, p. 384.
4. Lindegren, Carl C., and Rafalko, M., *Exp. Cell Res.*, **1**, 169 (1950).
5. Rafalko, M., and Lindegren, Carl C. (In press.)
6. Winge, O., *Compt. Rend. Lab. Carlsberg, Ser. Physiol.*, **21**, 77 (1935).

THE SPECIFICITY OF THE METHYL GREEN-PYRONIN STAIN FOR NUCLEIC ACIDS[1]

E. B. TAFT[2]

Department of Oncology, University of Kansas Medical School, Kansas City, Kansas

Received August 20, 1950

The problem of the demonstration of nucleic acids in tissue sections has long excited the interest of histologists and cytologists. The most satisfactory technique which has been developed to date for this purpose is that of Caspersson (2) which utilizes the absorption of ultraviolet light of specific wavelength to measure the quantities of desoxypentose and pentose nucleic acids in tissue sections. The main drawback of this technique is that it requires elaborate equipment not readily available to most investigators. An apparently adequate method for the demonstration and quantization of desoxypentose nucleic acid (5, 22) has been known for years, but no entirely satisfactory method for the ready histological localization of pentose nucleic acid has been developed except for that of Caspersson. Both types of nucleic acid stain with basic dyes, and many investigators have utilized this fact in the localization of these substances in tissue sections.

It has been suggested that methyl green, especially in the mixture of Pappenheim (16) or that of Unna, (26) stained chromatin selectively. Pappenheim (17) in his discussion of the mechanism of action of the staining solution of methyl green and pyronin suggested that the original theory of dye chemistry that basophilic substances stain with acid dyes as enunciated by Ehrlich and others is not necessarily the true state of affairs. He pointed to the triacid mixtures of Ehrlich (indulin, eosin and aurantia) and of Mallory (aniline blue, acid fuchsin and orange G) which stain nuclei as well as cytoplasm and other tissue constituents to prove his point. He said that there was no reason to suppose that a mixture of basic dyes might not result in differential staining as do mixtures of acid dyes. He thought that the methyl green stained chromatin selectively while the pyronin stained other basophilic and amphophilic substances in the cells more or less deeply.

[1] This investigation was aided by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

[2] Fellow of the National Research Council, 1948–1949. At present Research Fellow in Medicine at the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, at the Massachusetts General Hospital, Boston, Mass.

Subsequent papers describing the use of this stain were concerned largely with its use as a means of differentiation of plasma cells. Brachet (1) called attention to the fact that cells with pyroninophilic cytoplasm were those which were known to be rich in pentose nucleic acid such as those of the liver, pancreas and sea urchin eggs. With the isolation of an enzyme said to be specific for the degradation of pentose nucleic acid, ribonuclease (8), he felt that it definitely could be shown that the basophilia or pyroninophilia of the above cells represented pentose nucleic acid.

In 1947 Pollister and Ris (19) presented evidence which they believed confirmed this opinion. ". . . there are two remarkable basic dyes, pyronin and methyl green, which, as Brachet has demonstrated, have an astonishing ability to distinguish between the two nucleic acids. The reason for this is so far unknown, but we have been able to confirm the fact by staining isolated chromosomes and nuclei of known composition. The two dyes have completely different absorption maxima . . . and the ratio of extinction of these two wavelengths can be used to measure the relative amounts of the two nucleic acids."

In 1949 Pollister and Leuchtenberger (18) said that they had found a ". . . considerable residue of stainable substance in nuclei and cytoplasm after all nucleic acids and nucleotides have been removed chemically . . ." when using such dyes as pyronin B, pyronin Y or basic fuchsin. They were of the opinion, however, that in tissue fixed in acetic alcohol, ". . . purified methyl green is highly specific for desoxypentose nucleic acid." Thus Pollister would seem not to have kept his high opinion of the specificity of pyronin and methyl green as expressed in 1947.

One of the more important disadvantages of this stain, which had resulted in decrease of its use, is that it is variable and does not yield reproducible results. This problem has been reviewed recently, and a standardized stain has been recommended which in the hands of this investigator and his colleagues has given similarly good results at all times (25). The basis of the technique is an aqueous solution of dyes of the type originally described by Pappenheim (16) and revived by Scudder (21).

OBSERVATIONS ON FIXED TISSUE SECTIONS

Materials and methods.

In the light of the previous comments it seemed advisable to attempt to determine what is stained by pyronin and methyl green with the technique discussed in detail

in a previous publication (25). In part methods of control of specificity were used which had been described by other workers and in part methods were evolved for further realization of possible specificity. All the experiments reported below were made with purified methyl green and pyronin Y[1] in aqueous solution alone or in the combination and technique summarized here:

Dissolve 0.5 gm purified (by repeated extraction with chloroform in the dry state) methyl green and 0.1 gm pyronin Y in 100 cc hot distilled water. Store in amber, glass-stoppered bottles.

Sections on slides are hydrated and stained in above solution for three to five minutes. They are then rinsed briefly in distilled water and blotted almost dry with smooth filter paper. Sections are differentiated in "100 per cent" tertiary butyl alcohol (three parts tertiary butyl alcohol to one part of absolute ethyl alcohol (23)) for one to two minutes, cleared in xylol and mounted in permount.

Most of the observations were made on tissues fixed in absolute ethyl alcohol or in Carnoy's fluid (six parts absolute ethyl alcohol to three parts of chloroform to one part glacial acetic acid), but some were made on tissues fixed in neutral, aqueous formalin because fresh tissues were not available. Tissues were dehydrated by a graded alcohol-cedarwood oil technique and embedded in a mixture of beeswax and paraffin. Sections were cut at six microns, fixed to slides by albumin-glycerin and water and dried at 41° C. All observations reported below were controlled by staining a standard slide, untreated except for hydration, at the time the experimental slides were stained.

One of the simpler approaches to the problem of specificity is to utilize methods which will remove from tissue sections one or more of the components which are being studied. The method originally described by Schneider in which boiling trichloracetic acid is used to extract both pentose and desoxypentose nucleic acids from tissues has been adapted to tissue sections and used by various workers (4, 19) to remove all nucleic acid from the sections. This has been shown by ultraviolet absorption techniques to be a complete removal of such substances even from fixed tissue. In the present work a 5 per cent solution of trichloracetic acid heated to boiling (approximately 90° C) was used to extract the tissue sections on slides, usually for a period of fifteen minutes.

Molar sodium chloride has been said to specifically dissociate desoxypentose nucleoprotein (15) whereas it is said not to dissolve pentose nucleoprotein. Such a solution was used to extract tissue sections on slides.

Enzymes have been described by Kunitz (8, 9) which are said to be specific for the degradation of pentose nucleic acid and of desoxypentose nucleic acid. Although the exact action of these enzymes on nucleic acids is not completely understood, it has been shown that each will remove all detectable corresponding nucleic acid from tissue sections. The preparation of a "protease-free" ribonuclease was described by McDonald (13) and some of the reactions and the more satisfactory concentrations of enzyme were discussed in detail by Stowell and Zorzoli (24). Such an enzyme was prepared from beef pancreas (8) and used in the following technique:

[1] National Aniline Dyes were used in these experiments: Methyl green (ethylated hexamethyl pararosanilin) Cert. No. NG 26, Dye Cont. 85 per cent and Pyronin Y, Cert. No. NP 10, Dye Cont. 48 per cent.

Stock Solution:

Make up a solution of purified ribonuclease in physiological saline, 10.0 mgm/10 cc. Store in refrigerator at less than 5° C in glass-stoppered bottle. At time of use dilute 1: 10 with distilled water so that the final concentration will be 0.1 mgm enzyme per cc.

Technique:

1. Bring sections on slides to water.
2. Wipe off slide except immediately around section and place glass ring around section after coating both flat surfaces of the ring with vaseline. (15 × 3 mm glass rings been found to be quite satisfactory; however, plastic or rubber rings might work as well.)
3. Put approximately 0.3 cc of diluted ribonuclease solution over section in ring and cover with coverslip.
4. Incubate slide in oven at 55–65° C for 1 hour.
5. Incubate control slide in 1: 10 physiological saline at the same time.
6. Rinse slides in distilled water. (Vaseline may be removed with rag or by running slides up to xylol and back to water.)
7. Stain as desired.

Although Stowell and Zorzoli recommend the use of this enzyme in solution in distilled water, the enzyme was made up in saline for storage purpose since it was thought that the presence of non-toxic inorganic ions would help to maintain the stability of the enzyme. It is known that most of the pyroninophilic substance in cells is removed by incubation of tissue sections in physiological saline at 60° C, but it was found that little more of such material was extracted from tissue sections by diluted physiological saline than by distilled water. This technique was developed so that a standard amount of enzyme might be used on each section. If a larger volume of enzyme solution were used repeatedly, activity would gradually diminish in a way that could not well be standardized. The activity of the enzyme was well maintained for some two-three weeks under the above conditions.

Crystalline desoxyribonuclease[1] was used in an original technique derived from the observations of McCarty (12):

Stock solutions:

1. M/10 Hydrochloric acid.
2. M/10 Sodium veronal (2.06 gm/100 cc distilled water).
3. 3 M/100 Magnesium sulfate (0.61 gm $MgSO_4 \cdot 7\ H_2O$/100 cc distilled water). Add 0.1 gm gelatin and dissolve with heat.
4. To make M/40 veronal buffer at pH 7.5 with 3 M/1000 Mg^{++} and 0.01 per cent gelatin as recommended by McCarty:

10.05 cc M/10 HCl (1.)
14.95 cc M/10 Sodium veronal (2.)
10.00 cc $MgSO_4$ and gelatin solution (3).
65.00 cc Distilled water.

Test pH; should be 7.5.

[1] Purchased from the Worthington Chemical Co.

5. Stock desoxyribonuclease solution:

a. Dissolve approximately 1.0 mgm desoxyribonuclease in 4.95 cc distilled water. Check concentration of protein in solution according to Kunitz (9) formula using Beckman Ultraviolet Spectrophotometer:

Optical density = approximately 1.2/mgm protein/ml.

b. Make up to 15 cc with 10.05 cc undiluted buffer mixture in proportions as above (4. without distilled water). Store in refrigerator at less than 5° C in glass-stoppered bottle. At time of use dilute with distilled water so that the final concentration of desoxyribonuclease will approximate 1 mgm/30 cc. (McCarty recommends 3 mgm/100 cc.)

Technique:

1. Prepare slides as indicated for ribonuclease technique.
2. Place approximately 0.3 cc of diluted enzyme over section in ring and cover with cover slip.
3. Incubate at 37° C for 30 mins. (Tissues which have been fixed in stronger fixatives than absolute alcohol or Carnoy 6: 3: 1 may require 2–4 times as long a period of incubation.)
4. Control slide is incubated in dilute buffer at the same time.
5. Rinse slides and stain as desired.

Observations were made of the effect on the staining reaction of submerging sections in boiling water. Since Pollister and Leuchtenberger (18) expressed the opinion that the changes observed as a result of such treatment were perhaps caused by changes in the state of polymerization of desoxypentose nucleic acid, another method which might affect the state of polymerization was employed. This consisted of prolonged immersion of the tissue sections in solutions of high and low pH as outlined under Table I.

Preliminary observations were made almost entirely on sections of mouse liver. These observations were subsequently extended to include cartilage matrix, bone matrix, keratin, thyroid colloid, mucus, mast cell granules, necrotic tissue (both infarct and caseous necrosis), and amyloid. The latter two were available in formalin-fixed blocks. The others were fixed in Carnoy 6: 3: 1, absolute alcohol and neutral, aqueous formalin.

RESULTS

The results obtained on liver tissue sections are reported in Table I. Observations made with the combined stain and with individual dyes are essentially the same as those reported previously (25). As was expected, when the sections were extracted with hot trichloracetic acid, there was no evidence of staining in any of the liver cells or structural components of the liver. This would suggest that the staining of tissue components by this solution of dyes is effected by combination of the dyes with nucleic acids.

As can be seen in Table I, desoxyribonuclease appeared to remove quite specifically affinity of nuclei for methyl green and ribonuclease the pyroninophilia of the sections. The observations recorded above suggest that

TABLE I

Staining Reaction of Liver Parenchymal Cell Components Under Varying Circumstances.

Pretreament	Stain	Nuclear Chromatin	Nucleolus & Cytoplasmic Granulations	Other Liver Tissue Components not Nuclear
None	MGP	Blue	Rose-pink	Pale pink-tan to colorless
None	MG	Green-blue	Very pale green to colorless	Very pale green to colorless
None	P	Rose-pink	Rose-pink	Pale pink to colorless
pH 3, 1°[1]	MGP	Blue	Pink-rose	Pale pink-tan to colorless
pH 3, 24°	MGP	Blue-green	Pink	Pale pink-tan to colorless
pH 11.7, 1°[2]	MGP	Blue-green	Pink to pale pink	Pale tan to colorless
pH 11.7, 24°	MGP	Blue-green	Colorless	Very pale tan to colorless
Molar NaCl, 20′[3]	MGP	Pale grey-green	Colorless	Very pale tan to colorless
Boiling H_2O, 5′[4]	MGP	Lavender-blue	Pink	Pale tan to colorless
Boiling H_2O, 10′	MGP	Pale lavender-blue	Pale pink	Pale tan to colorless
Boiling H_2O, 15′	MGP	Pale lavender	Pale pink	Pale tan to colorless
Boiling H_2O, 30′	MGP	Pale lavender	Pale pink to colorless	Very pale tan to colorless
Hot 5 per cent TCA, 15′[5]	MGP	Colorless	Colorless	Colorless
PNA-ase, 63° C, 1°[6]	MGP	Green-blue	Colorless	Pale tan to colorless
DNA-ase, 37° C, 30′[7]	MGP	Colorless	Pink-rose[8]	Pale pink to colorless

staining with these dyes is the result of the presence of nucleic acids in the tissue sections, and further that affinity for methyl green is a property of

[1] 10 per cent glacial acetic acid in distilled water with enough dilute sodium acetate to raise pH to 3.0 on Beckman pH meter.
[2] Approximately 0.001 N NaOH in 70 per cent alcohol has pH 11.6–11.7.
[3] 5.85 gm NaCl made up to 100 cc with distilled water.
[4] Boiling water in beaker on hot plate (approximately 100° C).
[5] Boiling 5 per cent trichloracetic acid on hot plate (approximately 90° C).
[6] Ribonuclease used according to technique described.
[7] See accompanying text. Desoxyribonuclease used according to technique described.
[8] Some decrease in pyroninophilia noted in controls as well.

nuclear chromatin and hence of desoxypentose nucleic acid, and that affinity for pyronin Y is the property of nucleoli and cytoplasmic granulations, hence of pentose nucleic acid.

In an attempt to create a situation in which depolymerization might result to correlate the findings and theories of Pollister and Leuchtenberger who expressed the opinion that the affinity of desoxypentose nucleic acid for methyl green was an expression of the state of polymerization of the acid, sections were soaked in acid and alkaline solutions. No change was noted in either case in the staining reaction of the nuclei, but the pyroninophilia of the sections was markedly decreased more prominently in the alkaline bath. The observation of Pollister and Leuchtenberger and of Unna before them that a decrease in affinity for methyl green occurs when sections are immersed in boiling water was confirmed. One further observation was made which they did not report: the tissues also lost much of their pyroninophilia. It would seem that perhaps both nucleic acids may be altered or extracted by hot water and that pentose nucleic acid is the more labile.

The results with molar sodium chloride were most unexpected. The nuclear stain was admittedly greatly changed after soaking the sections in salt solution, but the pyroninophilia of the sections had completely disappeared. This suggests that the observation that desoxypentose nucleoprotein is dissolved by molar sodium chloride in contrast to pentose nucleoprotein may be a matter of proportion, and that given an enormous excess of salt solution as in this experiment, both nucleoproteins may be altered or extracted.

It seemed advisable to confirm some of these observations by the use of the Feulgen reaction. The technique of Stowell (22) was used for these experiments, the results of which are recorded in Table II. It can be seen that extracting the section with boiling 5 per cent trichloracetic acid destroys the ability of the desoxypentose nucleic acid to colorize leucofuchsin. This same ability is demonstrated by the enzyme desoxyribonuclease. It is most interesting that the nuclear chromatin was so pale after the sections were soaked in solutions at high and low pH since no alteration in coloration with methyl green could be detected under similar conditions. As had been reported by Pollister and Leuchtenberger, no diminution in the Feulgen reaction of the nuclei was found after soaking sections in boiling water.

The observations were then carried to other types of tissues and tissue components with the results noted in Table III. There are three observations which stand out in this table. In the first place, the fact that cartilage matrix is colored by methyl green in aqueous solution has not been commen-

TABLE II

Feulgen[1] Reaction of Liver Parenchymal Cell Components Under Varying Circumstances.

Pretreatment	Nuclear Chromatin	Nucleolus and Cytoplasm
None	Fuchsin	Green
pH 3, 24°	Lavender	Green
pH 11.7, 24°	Pale fuchsin	Green
Boiling H_2O, 10′	Fuchsin	Green
Hot 5 per cent TCA, 15′	Green	Green
PNA-ase, 1°	Fuchsin	Green
DNA-ase, 30′	Green	Green

ted on previously. The fact that it stains rose-red in a mixture of methyl green and pyronin has been noted before. That it loses its usual color after extraction with hot trichloracetic acid is also of interest.

The fact that thyroid colloid stains lavender from the solution either of the mixture of stains or of methyl green alone prompted the observations on the purification of methyl green reported in the review of this technique mentioned previously (25). In contrast to reports of other workers, with this technique it seems that thyroid colloid stains selectively with the methyl violet released by the breakdown of methyl green in aqueous solution. It stains very slightly if at all with pyronin.

The third observation which has been commented on infrequently is that mast cell granules appear to have a marked affinity for pyronin which is not removed by treating the section with ribonuclease. The granules also behave like cartilage matrix in that they stain blue-green from a solution of methyl green alone.

In the section of bone examined, nothing colored with pyronin except the bone matrix. This suggests that the decalcification procedure must extract most of the pentose nucleic acid from the tissue. The chromatin in this section stained a peculiar blue-grey somewhat similar to, but darker than that found after treatment of sections with molar sodium chloride. In this tissue as in the necrotic tissues which did not stain at all, it seemed fruitless to make further observations. The alterations in staining observed with keratin and mucus can hardly be considered significant. The fixation of the spleen containing amyloid was so poor that the value of the observations is doubtful, but they are recorded as observed.

[1] As modified by Stowell (22), with fast green counterstain.

TABLE III

Staining Reaction of Various Normal and Pathological Tissue Constituents Under Varying Circumstances.[1]

Pretreatment	Stain	Cartilage Matrix	Keratin	Thyroid Colloid	Mucus	Mast Cell Granules	Amyloid[2]
None	MGP	Rose-red	Pale lavender to colorless	Lavender-pink	Pale pink-tan to colorless	Rose-red	Pale pink-grey
None	MG	Blue-green	Pale lavender to colorless	Lavender-green	Colorless	Blue-green	—
None	P	Rose-red	Colorless	Very pale pink to colorless	Pale pink-tan to colorless	Rose-red	—
Hot 5 per cent TCA, 15′	MGP	Pink-tan	Pale lavender-tan	Lavender-pink	Colorless	Colorless	Colorless
PNA-ase, 1°	MGP	Rose-red	Pale lavender to colorless	Lavender-pink	Colorless	Rose-red	Pale pink-grey
DNA-ase, 30′	MGP	Rose-red	Pale lavender to colorless	Lavender-pink	Colorless	Rose-red	Pale pink-lavender

[1] Bone fixed in Carnoy (6: 3: 1) and decalcified 2 hours in 5 per cent formic acid showed pyroninophilia only in the bone matrix so that further observations were not made other than a simple methyl green-pyronin stain. Infarct necrosis, mouse liver, and caseous necrosis, human lymph node, both fixed in 10 per cent formalin, did not stain at all.

[2] Human spleen, far advanced pulmonary tuberculosis, fixed in formalin and stored for three years.

OBSERVATIONS ON PURE COMPOUNDS AND CELLULAR COMPONENTS

Materials and methods.

To observe pure substances with histological techniques is somewhat less simple perhaps than to make observations on tissue sections. Two methods suggest themselves: to mix the substance in question with a solution of gelatin and then to fix and embed blocks of this gelatin. Observations could be made on sections from such blocks. A second method which has been used extensively by Gomori (6) is to mix the substance in question with serum and to write with this mixture on glass slides.

The following techniques were devised:

1. Gelatin embedding.

A twenty per cent solution of ashless gelatin (Eastman Purified Calfskin Gelatin) was made in physiological saline. Equal quantities of this were mixed with portions of approximately one per cent solutions of commercial yeast nucleic acid and thymus nucleic acid in physiological saline. The gelatin was hardened in stenders in the refrigerator and blocks approximately $1 \times 1 \times 0.3$ cm were fixed in chilled 10 per cent aqueous formalin. (Blocks fixed in chilled 95 per cent alcohol and in chilled acetone shrivelled and became so hard that they could not be sectioned.) The blocks were left in formalin for approximately 18 hours and then were dehydrated in graded chilled alcohols beginning with 50 per cent. In 95 per cent alcohol they were brought to room temperature and the final dehydration and clearing were done at room temperature. Paraffin embedding and sectioning were done without difficulty in the usual manner.

Suspensions of nuclei, mitochondria, microsomes and the supernatant left from centrifugation of homogenized liver tissue (preparations were made by a technique similar to that described by Claude (3) for tumor tissue) were suspended in gelatin in a manner similar to that described above. Blocks of this gelatin were fixed in chilled 10 per cent formalin, dehydrated and embedded in paraffin. Smears of nuclear fractions as well as of the mitochondrial and microsomal fractions were likewise made. These were allowed to dry and then were fixed by immersion in 95 per cent alcohol for at least five minutes.

2. Solutions in Serum:

Human serum, free from apparent fat and hemolyzed blood, was used in these preparations. Approximately one per cent solutions of commercial yeast nucleic acid and thymus nucleic acid were made in serum. A similar solution, though somewhat more dilute, of mouse liver pentose nucleic acid and one of mouse liver desoxypentose nucleic acid were made. Solutions of commercial protamine, adenylic acid and guanylic acid were likewise made. With a clean steel pen a linear mark was made upon a number of glass slides with each of these solutions and with a control of plain serum. The slides were allowed to dry. Then the serum was fixed as follows: the slides were immersed for one hour in absolute ethyl alcohol; one hour in xylol at 60° C; and a final rinse in absolute alcohol. They were stored in a dry state prior to staining. (If the slides were not put through xylol at 60° C as suggested by Gomori (6) the serum frequently did not remain attached to the slides.)

RESULTS

Nuclei and cytoplasmic fractions embedded in gelatin did not stain as well as smears made from these components. In smears the nuclear chromatin stained blue and the nucleoli rose-pink as usual. The mitochondria stained pink as did the microsomes. In the gelatin-embedded fractions, the mitochondria and microsomes stained bright pink, but the supernatant stained only a pale pink or not at all. The gelatin stained very faintly blue-grey or not at all when sections of plain gelatin were tested. The blocks of gelatin containing commercial nucleic acids did not stain consistently nor differentially.

When slides with serum writing were stained in pyronin-methyl green solution, the commercial yeast nucleic acid stained a clear rose-red. The commercial thymus nucleic acid stained in a mottled manner –blue-green flecks on a pale pink-lavender background. The control serum usually stained a pale pink-lavender or pale lavender. The mouse pentose nucleic acid stained a clear rose-pink; the mouse desoxypentose nucleic acid stained a pale blue-lavender. The solutions of protamine, adenylic acid and guanylic acid did not change the usual color of the serum. After immersion in either boiling trichloracetic acid or in boiling water for fifteen minutes, the marks of serum all stained pale lavender.

DISCUSSION

The mode of action of this stain has been the subject of considerable discussion and was recently investigated in detail by Pollister and Leuchtenberger (18). Many of the points brought out by these authors are of interest. Some of the observations reported in this paper tend to contradict their hypothesis that the staining of desoxypentose nucleic acid with methyl green depends to a large extent on the state of polymerization of the nucleic acid. According to Gulland and Jordan (7) desoxypentose nucleic acid is depolymerized at pH 3.0 and at pH 12.0. Although test tube observations cannot always be correlated to histologic observations it would seem likely that if the state of polymerization were the important factor concerned in the reaction of the desoxypentose nucleic acid with methyl green, the chromatin would not stain after sections had been soaked for twenty-four hours at pH 3.0 and only very slightly, perhaps, after pH 11.7. Instead, there is little evidence that the staining reaction with methyl green is affected. The fact that the pyroninophilia of the section is completely removed by treat-

ment at pH 11.7 suggests that pentose nucleic acid is dissolved not just depolymerized. This thesis was tested by covering a section with a thin film of celloidin prior to soaking the section at the high pH. The coloration with pyronin was as completely removed under these circumstances as before. The decrease in intensity of the Feulgen reaction of the nuclei after treating sections at low and high pH is suggestive evidence that the desoxypentose nucleic acid was lost from the fixed tissue sections. The aldehyde in the desoxypentose is not hydrolyzed in alkaline solution and only minimally at pH 3.0, so that this cannot be the explanation for decrease in intensity of the Feulgen reaction. If Pollister and Leuchtenberger are correct that the effect of hot water is depolymerization, it seems strange that another reaction, known to cause depolymerization of desoxypentose nucleic acid in test tubes and which causes a decrease of stain by the Feulgen technique, should not give results with the pyronin-methyl green stain similar to those found with the hot water treatment.

Methyl green is a bivalent cation in solution and has been said to differ from most other basic and acidic dyes in this respect (14). It may well be that this is the reason why the dye has an affinity for chromatin. It hardly seems, however, that it is because desoxypentose nucleoprotein has free orthophosphoric acid groups that this dye is attracted to chromatin, for pentose nucleoprotein has similar free orthophosphoric acid groups. It may of course be possible that differences in structural relationships may account for differences in availability of these acid groups and hence of the staining characteristics of the nucleic acids as has been recently suggested by Kurnick and Mirsky (10, 11).

The question of residual staining after extraction with hot trichloracetic acid concerned Pollister and Leuchtenberger (18) and was one of the main reasons given for discarding the theory that pyronin is perhaps a specific stain for pentose nucleic acid. From the results reported in Table I, it is suggested that perhaps the reason for their observed residual staining is not that there are substances in liver cells other than pentose nucleic acid which stain with pyronin, but rather that their technique of use of this combination of dyes may be at fault. With the modified Scudder technique used in these experiments residual staining after extraction with hot trichloracetic could not be detected.

The problem of the specificity of this staining reaction for the demonstration and differentiation of desoxypentose and pentose nucleic acids obviously has not been completely settled by this investigation. A number of aspects of the problem have become apparent, however. It seems reasonably cer-

tain that methyl green, when used by itself or in combination with pyronin in an aqueous solution such as described above, stains nuclear chromatin in a manner comparable to its demonstration by the Feulgen technique. That this staining reaction represents the content of desoxypentose nucleic acid in the nucleus has been suggested by the work of Pollister and Ris (19) and Pollister and Leuchtenberger (18). Whether pyronin is as specific a stain for pentose nucleic acid is not as certain. Again, however, in such a cell as the liver parenchymal cell, it seems that the pyroninophilic material in the nucleolus and cytoplasmic granulations represents pentose nucleic acid.

The two valuable tools first isolated and described by Kunitz, ribonuclease and desoxyribonuclease, offer much help in this problem. As has been obvious in the work reported on the examination of liver parenchymal cells, these enzymes appear to remove all pyroninophilic material from the liver cells on the one hand and all substances with affinity for methyl green on the other. This further supports the thesis that in the liver parenchymal cell, at least, the stains are specific for the demonstration of pentose nucleic acid and desoxypentose nucleic acid.

The accumulated evidence for the probable specificity of these dyes is further enhanced by the reactions described for the pure substances dissolved in serum and stained on slides. However, with other tissues than liver parenchymal cells such as cartilage, bone, thyroid colloid, and mast cell granules and pathological substances, the problem becomes much less simple. It is obvious that the coloration by pyronin with the combined dyes, or by each in simple solution, of cartilage matrix and the granules of mast cells cannot be explained by the content of nucleic acid in these substances. The staining reactions are undoubtedly the result of their high content of complex polysaccharide-sulfuric acid substances. When it becomes necessary to work with tissues containing such substances as have been enumerated, very careful control of the reactions must be used such as appropriate enzymes and extraction with trichloracetic acid, even though the latter procedure appears to extract much of the stainable material from the cartilage matrix and mast cells, as well as nucleic acid from the sections.

SUMMARY AND CONCLUSIONS

The question of the specificity of methyl green and pyronin for the demonstration and differentiation of nucleic acids in tissue sections is considered in some detail. Three approaches to the problem are presented. In the first

only the reactions in the liver parenchymal cell of the mouse are described. There it is apparent that the stains differentiate the two types of nucleic acid. Methyl green appears to have an affinity for desoxypentose nucleic acid in the nuclear chromatin and pyronin for pentose nucleic acid in the nucleoli and cytoplasmic granulations. Methods of control of the specificity of this reaction involving the use of extraction of sections with trichloracetic acid which completely removes nucleic acid from tissues, the use of enzymes apparently specific for the degradation of nucleic acids, and the use of the Feulgen reaction for the demonstration of desoxypentose nucleic acid are discussed.

The second approach involves the study of various natural and pathological cellular constituents and products: cartilage matrix, mast cell granules, thyroid colloid, keratin, mucus and amyloid. The reactions of these substances are presented, reasons for their coloration are discussed, and control of their reactions is outlined.

Finally by the use of purified substances such as yeast nucleic acid, thymus nucleic acid, mouse liver nucleic acids, basic proteins and nucleotides the specificity of this stain is further defined. Methods for the demonstration of such substances on a histological basis are presented. Their reactions are discussed.

The theories of Pollister and Leuchtenberger to explain the apparent specificity of methyl green for nuclear chromatin are discussed. Evidence in disagreement with their theory that the state of polymerization of desoxypentose nucleic acid is the responsible factor is presented. It is suggested that the presence of orthophosphoric acid groups can hardly be the mechanism for this stain. It is concluded that the question of the specificity of these stains for the demonstration of nucleic acids in tissue sections has not been completely decided by these experiments. The evidence presented, however, is highly suggestive that, under certain cirumstances and with proper controls, these stains apparently localize the two types of nucleic acid found in tissue sections.

REFERENCES

1. Brachet, J., *Compt. rend. Soc. biol.*, **133**, 88 (1940).
2. Caspersson, T., *J. Roy. Micr. Soc.*, **60**, 8 (1940).
3. Claude, A., *J. Exptl. Med.*, **80**, 19 (1944).
4. Di Stephano, H. S., *Chromosoma*, **3**, 282 (1948).
5. Feulgen, R., and Rossenbeck, H., *Z. physiol. Chem.*, **135**, 203 (1924).
6. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **68**, 354 (1948).
7. Gulland, J. M., and Jordan, D. O., *Symposia of the Society for Exptl. Biol.*, **1**, 56 (1947).
8. Kunitz, M., *J. Gen. Physiol.*, **24**, 15 (1940).
9. —— *Science*, **108**, 19 (1948).

10. Kurnick, N. B., *J. Gen. Physiol.*, **33**, 243 (1950).
11. Kurnick, N. B., and Mirsky, A. E., *J. Gen. Physiol.*, **33**, 265 (1950).
12. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
13. McDonald, M. R., *J. Gen. Physiol.*, **32**, 39 (1948).
14. Michaelis, L., *Cold Spring Harbor Symposia*, **12**, 131 (1947).
15. Mirsky, A. E., *Cold Spring Harbor Symposia*, **12**, 143 (1947).
16. Pappenheim, A., *Virchows Arch. path. Anat. Physiol.*, **157**, 19 (1899).
17. —— *Folia haem.*, **6**, 51 (1908).
18. Pollister, A. W., and Leuchtenberger, C., *Proc. Natl. Acad. Sci.*, **35**, 111 (1949).
19. Pollister, A. W., and Ris, H., *Cold Spring Harbor Symposia*, **12**, 147 (1947).
20. Schneider, W., *J. Biol. Chem.*, **181**, 293 (1947).
21. Scudder, S. A., *Stain Technol.*, **19**, 39 (1944).
22. Stowell, R. E., *Cancer Research*, **6**, 426 (1946).
23. —— *Science*, **96**, 165 (1942).
24. Stowell, R. E., and Zorzoli, A., *Stain Technol.*, **22**, 51 (1947).
25. Taft, E. B., *Stain Technol.* (1951). (In press).
26. Unna, P. C., *Monatschr. prakt. Dermat.*, **35**, 76 (1902).

CYTOLOGICAL, CYTOCHEMICAL AND BIOCHEMICAL OBSERVATIONS ON SECRETING AND RESTING SALIVARY GLANDS[1]

LUIZ CARLOS U. JUNQUEIRA

Laboratório de Histologia e Embriologia, Faculdade de Medicina, Universidade de S. Paulo, Brasil

Received September 6, 1950

INTRODUCTION

A series of experiments was made in which the ducts of submaxillary glands in mice were ligated. It soon became evident that the ligated glands atrophied. Histologically these glands showed no degenerative processes and no secretion could be observed in the acinar and tubular portions. It became clear later that although atrophied, the tubular portions of ligated glands retain their capacity to secrete when properly stimulated. A gland in such a condition is called "resting gland" throughout this work and should have different metabolism than an actively secreting one. We believe that the study of ligated glands when compared with controls might give us a clue to some of the biological processes which are directly or indirectly related to submaxillary gland secretion. In this paper we present the results obtained applying histological, cytochemical and biochemical methods in the study of normal and ligated glands.

MATERIAL AND METHODS

Adult male albino mice from Adolfo Lutz Institute were used throughout the experiment. The ducts of glands of the right side were exposed by an incision in the skin of the cervical region in mice anesthetized with ether. Once dissected free from the vessels and nerves of the submaxillary glands, they were ligated with cotton thread. Left glands remained always as controls.

For histological observations glands were fixed in Helly's fluid for 6 hours embedded in paraffin and stained with Mallory's phosphotungstic hematoxylin, Mallory's trichrome and Heidenhain's iron hematoxylin. Secretory granules were conspicuously stained when Mallory's phosphotungstic hematoxylin was used. Mito-

[1] This study was aided by grants from the Rockefeller Foundation and Comissão de Pesquisa Científica da Universidade de S. Paulo.

chondria were stained using Mallory's phosphotungstic hematoxylin on sections of post-chromated glands.

Tests for protease and amylase activity previously described in the submaxillaries of adult males by Junqueira *et al.* (16, 15), were made on homogenates of ligated and control glands following the methods described by Pickford and Dorris (27).

Preparations for the study of alkaline and acid phosphatase were incubated according to the methods of Gomori described by Glick (12). Chilled alcohol or acetone fixation was employed. For alkaline phosphatase detection sections of alcohol fixed, paraffin embedded material were made, while frozen sections of acetone fixed material were used for acid phosphatase demonstration.

Controls for the acid phosphatase method were incubated with 0.01 M sodium fluoride and for the alkaline phosphatase technique without glycerophosphate or with glycerophosphate plus 0.01 M sodium cyanide.

Brachet's ribonuclease method was used to study the distribution of ribonuclease removable basophilia. Tissues were fixed in Helly's fluid and Brachet's technique as described by Stowell and Zorzoli (31) was followed. Incubation was done however for 6 hours at 37° C in a 0.15 M solution of monopotassium phosphate at pH 4.5 in order to avoid the proteolytic activity of crystalline ribonuclease described by Kleczkowski (20).

Millon's reaction was applied in order to detect proteins containing tyrosine. Distribution of disulfide groups bound to proteins was studied with the aid of Hopkin's nitroprusside reagent. Details of both these techniques have already been published (15).

Cytochrome oxidase distribution was studied applying the indophenol blue technique described by Moog (24). Keilin's work (18) suggests that the indophenol blue production is a reliable evidence of the presence of an enzyme or enzymes having the properties of cytochrome oxidase. Frozen sections (50 μ thick) of fresh control and ligated glands were incubated at 37° C in Nadi's reagent at pH 6.0. Controls were made with 0.3 N azide or 0.01 M potassium cyanide as inhibitors. Both these substances prevented color development.

Succinic dehydrogenase activity was studied in control and ligated glands by the rate of methylene blue discoloration in Thunberg tubes. To every tube we added 1 cm^3 of 0.9 per cent sodium chloride, 1 cm^3 M/15 phosphate buffer at pH 7.6, 0.5 cm^3 of 0.02 M sodium succinate, 0.1 cm^3 of 1/5000 methylene blue and 30 mg of minced glands. To prevent bumping the solutions were previously evacuated and kept under liquid vaselin in the ice box. The sodium chloride solution and the minced gland were deposited in the bottom of the tube, while the other solutions went into the sidearm. After evacuating the tubes and heating them to 37° C the solutions were mixed and collected in the bottom of the tube. The methylene blue reduction was measured photometrically in a Coleman Jr. spectrophotometer with 660 mμ wave length.

All histological and cytochemical observations were made on a minimum of 6 animals. Cytochemical tests were always done using animals between the 15th and 30th day after ligation. The cytochemical results obtained with the phosphatase techniques were checked biochemically on gland homogenates employing Binkley, Schank and Hoagland's (3) method adapted to be used in a 1 cm^3 cuvette in a Coleman Jr. spectrophotometer. Incubation was carried out at 37° C during one hour.

Lacassagne (23) demonstrated that the secretion of the cells of the tubular portion can be stimulated by administering androgens to mice. Based on this previous knowledge an androgen was injected in mice with ligated glands in order to discover if the ligated glands still retain the capacity of secreting when adequately stimulated. Ten animals whose gland duct had been ligated 15 days previously were separated in two groups, 5 remaining as controls, while the other 5 were injected on alternate days over a 10 day period with 2.5 mg of testosterone propionate.[1]

RESULTS

Histological and cytological considerations

No detailed description of the normal submaxillary glands will be given for it has already been published (32, 15). The submaxillary gland of the adult male mouse consists essentially of two types of structures, acini and serous tubules. The acinar cells are of the special serous type (30), have a strong basal cytoplasmic basophilia, probably produce amylase and contain mucoproteins (15). These acini are joined by short intercalated ducts to the serous tubules, which in turn empty into excretory ducts. We will describe here the aspects presented by glands when studied at different periods after ligation. Two or three days after this operation one can easily observe microscopically the beginning of gland atrophy, for a diminution of the diameter of the serous tubules and of the amount of secretory granules can be seen. This process attains its maximum between the 15th and the 30th days after the operation. At this time both tubular and acinar portions are greatly atrophied. The cells of the serous tubules which normally have a prismatic shape, now appear cuboidal. In their cytoplasm no secretory granules can be noticed. The cells of the acinous portion present no secretory granules and their cytoplasm is considerably reduced and does not stain with the methods used. The nuclei of these "resting" cells show no alterations that would lead us to suspect degenerative processes (figs. 2, 3, 4, 5). In glands which had been ligated for more than two months there is an attempt to return to the normal aspect and discrete quantities of secretory granules occur in the cells of the tubular portions.

When glands ligated 15 days previously are stimulated by repeated injections of androgens the tubular portions contain moderate amounts of secretory granules. This indicates that these glands, although with ligated duct, retain their capacity to secrete when properly stimulated (figs. 6, 7, 8, 9). The mitochondria present in the basal portion of the tubular cells are normally thread-like and abundant. In ligated glands they diminish

[1] The author is grateful to Ciba Co., for the testosterone propionate used.

to such an extent that they pratically disappear. In stimulated glands of animals injected with androgen a moderate quantity of these mitochondria reappears. The mitochondria of the acinar cells in normal glands have the form of short rods and are distributed throughout the cytoplasm. In ligated glands they disappear.

Cytochemical and biochemical observations

Proteins.—Millon's and Hopkin's reactions when applied to control glands showed the presence of tyrosine and protein-bound disulfide groups in the apical portion of the cells of the serous tubules (for more details see Junqueira *et al.* (15)). In ligated glands these reactions were negative.

Amylase and protease.—With the tests of Pickford and Dorris for protease and amylase a sharp decrease of these enzymes activity was observed in the ligated gland when compared with the controls.

Acid phosphatase.—This enzyme is present in the nuclei, and apical zone of the serous tubule cells of control glands. In ligated glands a positive reaction could be detected only in the nuclei. These histochemical results were confirmed with acid phosphatase determinations on homogenates of control and ligated glands (table I.) Observing the results obtained one can notice the sharp drop of acid phosphatase in ligated glands as compared with the controls.

TABLE I

Acid and alkaline phosphatase activity of 12 control and 12 ligated glands from mice ligated 25 days previously. Results in mg of phenol set free by 100 mg of tissue (wet weight).

	Controls	Ligated
Acid phosphatase	2.0 ± 0.28	0.42 ± 0.15
Alkaline phosphatase	2.0 ± 0.26	2.4 ± 0.4

Basophilia removable by ribonuclease.—Sections of control glands stained with toluidine blue present a basophilia dispelled by ribonuclease in the basal region of the cells of the acini and serous tubules (fig. 10). In ligated glands this basophilia is absent (fig. 11).

Alkaline phosphatase.—In preparations with Gomori's method this enzyme is present in control glands in the acinar portion, and in the connective tissue that surrounds the serous tubules (fig. 12).

In ligated glands one can observe a positive reaction in the above mentioned sites plus in the atrophied serous tubules. It is interesting to note that sections of ligated glands stain much more intensely than those of control

glands (fig. 13), yet determinations on homogenates show no great variation in the activity of this enzyme between ligated and control glands (table I).

The reaction in the peritubular connective tissue is a very strong one, and we suspect that the precipitate observed by other authors (25, 11) in the basal portion of the secretory tubule cells is due to diffusion phenomena. One of the main reasons to suppose so is the fact that no positive reaction can be observed in the basal portion of these cells when the cell is shrunken by fixation and there is a gap between it and the peritubular connective tissue (fig. 14).

Indophenol oxidase.—Sections of control and ligated glands cut and incubated at the same time developed a different color. The staining began in both glands after 3–6 minutes at 37° C, but the controls always stained a distinctly darker blue than the ligated glands. After 10 minutes incubation this difference becomes quite evident, particularly when the sections are observed under low magnification and with a red filter.

Succinic dehydrogenase.—The results obtained with the method used are expressed in the graph of fig. 1. Every point of this graph is a mean of 8

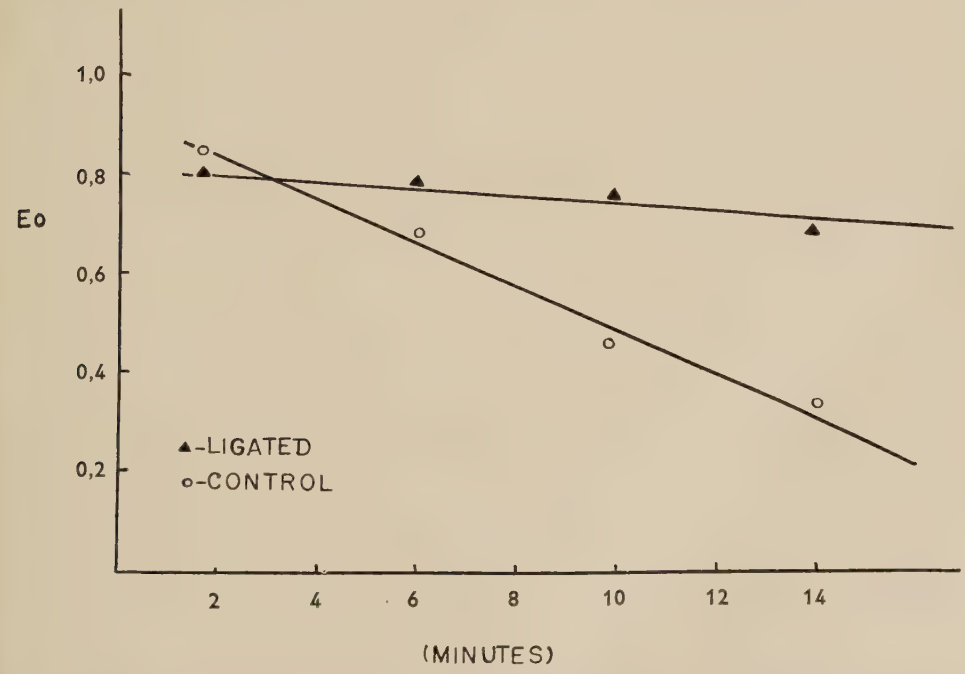


Fig. 1. Succinic dehydrogenase activity of control and ligated glands as measured by the rate of methylene blue discoloration. Every point is the mean of eight determinations.

determinations. A visible difference between the rate of discoloration of control and ligated glands can be observed. These results suggest a strong decrease of the succinic dehydrogenase activity in ligated glands. Although individual variations were observed, in all 8 experiments the rate of methylene blue reduction was always distinctly slower in tubes with ligated glands as compared with those containing controls. The fact that malonate prevents the methylene blue reduction and that without succinate no reduction can be observed are evidences that we are dealing with succinic dehydrogenase.

DISCUSSION

Cellular secretion is a complex process in which at least two main phenomena take place, that is synthesis of secretory granules and their expulsion. The synthesis of granules depends on the transport of the "raw material" from the extracellular fluid into the cell and the grouping of these substances.

Evidently these processes represent work and one of the main problems to be studied in cell secretion is the source of energy for such work.

There seems to be no doubt in the literature that submaxillary glands have a glycolytic pathway for carbohydrate metabolism (1, 2, 10, 26, 8). Data from Ferrari and Hoeber (10) suggest the participation of the cytochrome-cytochrome oxidase system in submaxillary gland metabolism. Re-

---

PLATE I

Explanation of figures.

Fig. 2. Section from a control submaxillary gland of an adult male. Observe developed tubular and acinar portions. Abundant secretory granules in the tubular cells. Hematoxylin and eosin. ×600.

Fig. 3. Section from a 15-day ligated gland from the same animal as in fig. 1. The tubular and acinar portions are atrophied. No degenerative alterations of the nuclei are visible. Secretory granules are absent. Hematoxylin and eosin. ×600.

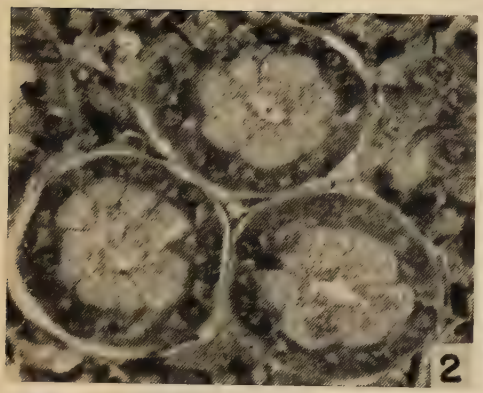
Fig. 4–5. The same as figures 2 and 3 with lower magnification. Observe how the excretory ducts are dilated in the ligated glands. The atrophy of the acini is so complete that they are hardly visible. Hematoxylin and eosin. ×150.

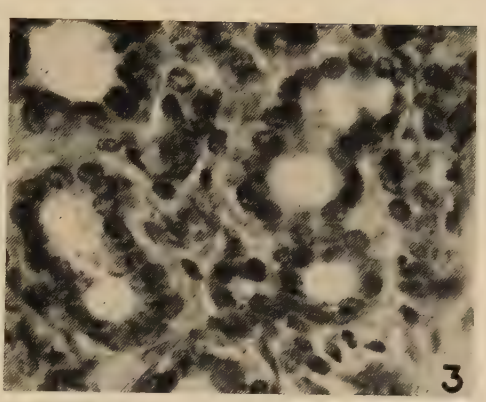
Fig. 6. Section from a control and ligated submaxillary gland. Both glands from the same animal. Observe secretory tubules (beneath) of control gland full of secretion. Ligated gland (above) with no secretion and dilated excretory ducts. Mallory's phosphotungstic hematoxylin. ×12.

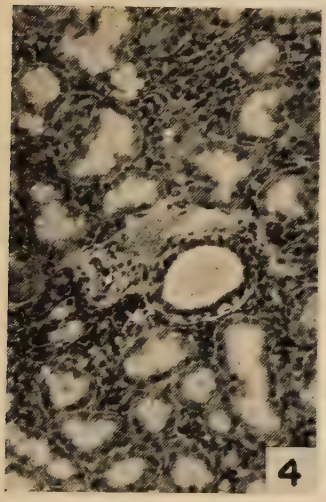
Fig. 7. Sections with higher magnification from the ligated gland of an androgen treated mouse. Observe moderate quantity of secretory granules in the tubular cells. The acini are hardly visible. Mallory's phosphotungstic hematoxylin. ×180.

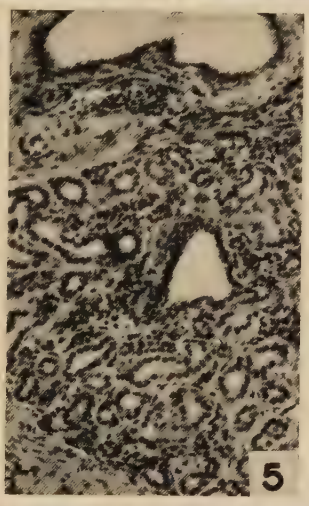
Fig. 8. The same from a control gland. Secretory tubules are very developed. Observe presence of acini. Mallory's phosphotungstic hematoxylin. ×180.

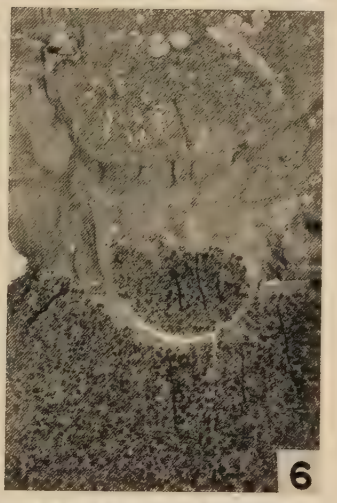
Fig. 9. The same from an animal that received 2.5 mg of testosterone propionate. Observe intense secretion in the control gland (beneath) and discrete secretory phenomena in the peripheral tubules of the ligated gland. Mallory's phosphotungstic hematoxylin. ×12.

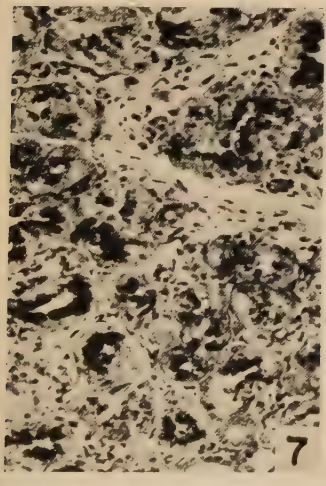

2

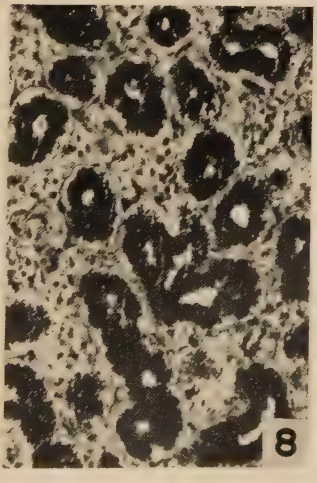

3


4

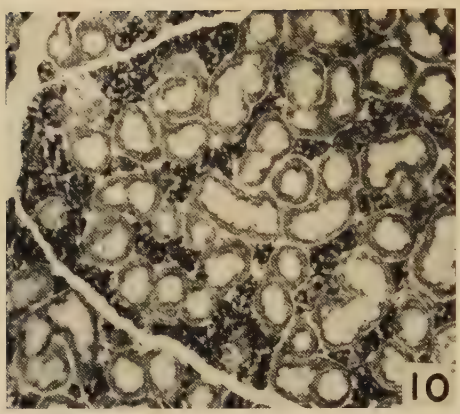

5

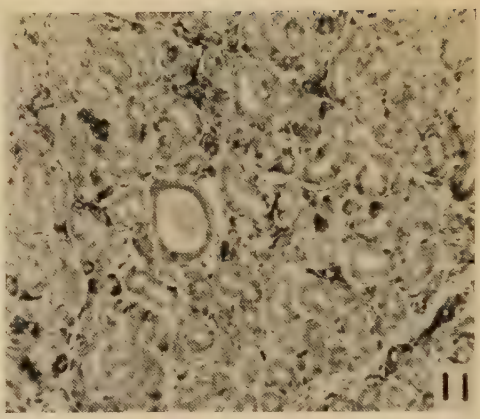

6

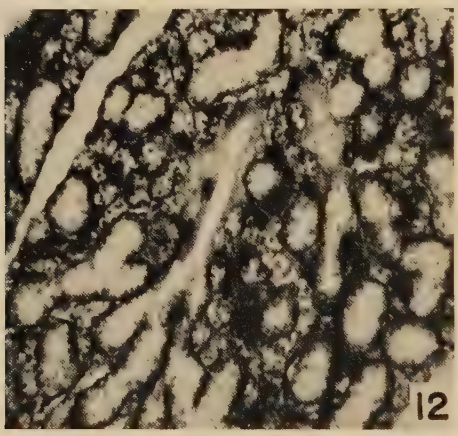

7

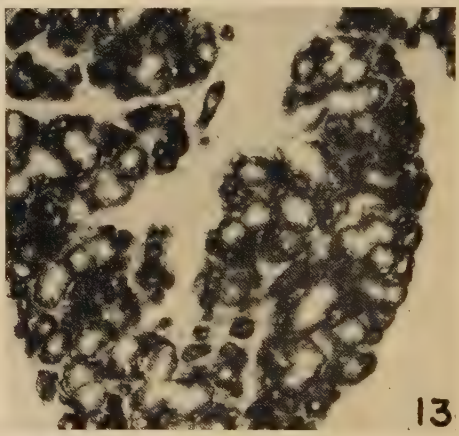

8

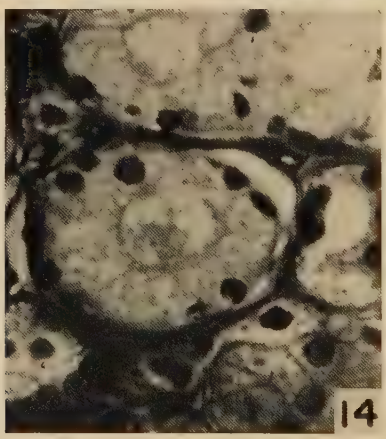
9


10


11


12


13


14

sults of Camis (4), Ferrari and Höber (10) and Northup (26) suggest the participation of energy-rich phosphorus bonds in the metabolism of these glands and it is of interest to remember here that in the rat one of the organs richest in adenosine triphosphatase is the submaxillary gland (9). To our knowledge evidence of the presence of Krebs' tricarboxylic acid cycle in submaxillary glands is missing.

With the methods used no clear insight into the processes concerned in secretion can be obtained but we hope in this and in subsequent papers, by comparing control and ligated glands, to get a clue to the nature of these processes and also those related to the basic phenomena of cell metabolism responsible for the maintenance of cell structure and life.

The facts that the cells of ligated glands present no degenerative processes, and that they are able to secrete when adequately stimulated, are evidence that they are alive and that possibly one of the main effects of ligation is to suppress cell secretion. If this be true we would have an adequate biological reagent to study differences between resting and working glands and processes related to cell secretion.

This approach to the study of cell metabolism has already been made by several authors notably Ferrari and Höber (10), Deutsch and Raper (8), Korr (22) and Stannard (29) who studied respectively, submaxillary glands stimulated pharmacologically and electrically, myometrium stimulated by oxytocin, and frog muscle stimulated by caffeine or electrically. Kochakian and Dontigny's work (21) on the enzymes of Selye's "endocrine kidney" seems to us a promising approach to the study of kidney metabolism related to urine secretion. Probably a submaxillary gland not under nervous or pharmacological stimulus is not a resting gland, for synthetic processes are very likely going on. We are under the impression that the ligated gland represents a purer "resting gland" than those already studied. It seems

---

PLATE II

Fig. 10. Section from a control gland. Strong ribonuclease-removable basophilia in the basal region of acinar and tubular cells. Toluidine blue stain. ×150.

Fig. 11. Section of a ligated gland stained with toluidine blue. Observe very faint basophilia present practically only in the cell nuclei. ×150.

Fig. 12. Section of a control gland. Observe strong reaction for alkaline phosphatase in the acini and principally in the peritubular connective tissue. Gomori's method. ×140.

Fig. 13. Section of a ligated gland. Observe strong alkaline phosphatase reaction in acinar and tubular cells. Excretory tubules with no activity. Gomori's method. ×140.

Fig. 14. Section of a control gland. Observe a pseudo-positive reaction for alkaline phosphatase to the right of the central tubule, while to the left where the cell base is detached from the peritubular connective tissue no reaction is observable. Gomori's technique. ×700.

also to be an interesting example in which to study metabolic phenomena related to maintenance of life and cell structure.

The results obtained with the indophenol oxidase technique suggests a participation of the cytochrome-cytochrome oxidase system in mice submaxillary gland secretion. The data obtained on succinic dehydrogenase are evidence for the presence of at least a part of Krebs tricarboxilic acid in mice submaxillary gland metabolism. These results were confirmed manometrically by Junqueira (14) for an increase of Q02 could be obtained adding succinate. Malonate inhibited this effect. The fact that the activity of this enzyme is considerably reduced in ligated glands as compared to controls suggests its participation in the secretory phenomena of these glands.

The disappearance of the mithochondria in the cells of resting submaxillary glands as compared to controls is more an indication of the participation of these elements in cell secretion (for a discussion on this subject see (7). These results are of interest if we remember that the mitochondria are very probably the site of cytochrome-oxidase, succinic dehydrogenase activity (13), fatty acid oxidation (19) and oxalacetic oxidase (28).

The disappearance of a positive reaction for tyrosine and protein-bound disulfide groups from the apical portion of the serous tubule cells is in our opinion cytochemical evidence for the lack of synthesis of secretory granules. The sharp decrease of amylase and protease activity confirms this point of view. Both these facts confirm the microscopical observations that secretory phenomena could not be demonstrated in ligated glands.

The correlation between ribonucleoproteins and protein synthesis postulated by Caspersson's school (5) has since then been the subject of several papers. The disappearance of all basophilia removable by ribonuclease in ligated glands suggests such a correlation.[1]

Our data on alkaline phosphatase summed up with those already published on the distribution of this enzyme in glands of adult and prepuberal mice (15) do not confirm Feyel-Cabanne's (11) report that this enzyme has a direct participation in the synthesis of the serous granules.

The pseudo-positive reaction observed in the basal portion of the serous tubule cells is probably an example of a limited diffusion of the precipitate.

The fact that sections of ligated glands stain much darker than those of controls would lead us to think that these glands have an increased alkaline phosphatase activity. Biochemical results with homogenates, however, prove

[1] After this paper was submitted to publication nucleic acids phosphorus determinations in control and duct ligated glands obtained by M. Rabinovitch in this laboratory, confirmed these findings.

that the activity of both glands does not differ as much as one might suppose from histochemical data.

These two facts serve to illustrate how results in histochemistry must be interpreted carefully.

The acid phosphatase on the contrary seems to have a role in the submaxillary gland activity, for homogenates of ligated glands present a dramatic decrease of activity when compared to controls. It is probable that the residual activity of this enzyme in the ligated glands is due to the enzyme present in the cell nuclei.

No study of the cell nuclei was made, but they very probably represent a good material in which to study nuclear phenomena related to cell secretion.

Observations on the adenosinetriphosphatase activity, creatine phosphate content and action of cyanide, azide, monoiodoacetic acid and fluoride, on the metabolism of ligated and control glands would be of interest. Some of these aspects are being studied in this laboratory.

It would also be of interest here to remark on the work of Katagiri and Higashijo (17) who on the basis of the different action of oestrogens on the uterus of normal mice and those in which the submaxillary glands had been extirpated, concluded that these glands have an endocrine function beside that of producing digestive enzymes. Unpublished experiments in this laboratory showed no difference between the genital organs of normal and submaxillary extirpated mice of both sexes. In addition to the macroscopical and histological observations, on females it was noted that the estrous cycle as followed by vaginal smears was the same in both groups.

SUMMARY

The ligation of the excretory duct of submaxillary glands of mice leads to an atrophy of their tubular and acinar portions. This leads to a reduction in size of the tubular and acinar cells, secretory granules and mitochondria could not be observed in these cells. The absence of secretory granules was confirmed cytochemically by the lack of tyrosine and protein-bound disulfide groups in the tubular and acinar cells.

Biochemically a sharp reduction in the content of protease and amylase could be observed. Hoping that a comparison of ligated with control glands might give a clue to some processes related to cell secretion, some histological and biochemical observations were made on control and ligated glands with the following results:

1. Indophenol oxidase and succinic dehydrogenase (studied with the Nadi's reagent and Thunberg method) activity was diminished in ligated glands as compared to the controls.

2. Biochemically and cytochemically a sharp decrease of acid phosphatase activity but no significant change in alkaline phosphatase was observed after duct ligation.

3. Basophilia removable by ribonuclease disappeared almost completely in the ligated glands.

It is suggested that acid phosphatase, indophenol oxidase, succinic dehydrogenase and basophilia removable by ribonuclease probably play a role in submaxillary cell secretion.

The significance of the results obtained is discussed.

REFERENCES

1. Anrep, G. V., and Cannan, R. R., *J. Physiol.*, **56**, 248 (1922).
2. Bergonzi, J., and Boleato, U., *in* Ferrari, R. and Höber, R., 1933, *loco cit.*
3. Binkeley, R., Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, **156**, 253 (1944).
4. Camis, M., *Arch. Int. Physiol.*, **22**, 345 (1924).
5. Caspersson, T., *Soc. Exp. Symp. Univ. Press*, **1**, 127 (1947).
6. Commoner, R., *J. Cell. Comp. Physiol.*, **13**, 121 (1939).
7. De Robertis, E. D. P., Nowinski, W. W., and Saez, F. A., General Cytology, Saunders Co., Philadelphia, p. 89, 1948.
8. Deutsch, W., and Raper, H. S., *J. Physiol.*, **92**, 439 (1938).
9. Du Bois, R. P., and Potter, V. R., *J. Biol. Chem.*, **150**, 185 (1943).
10. Ferrari, R., and Höber, R., *Pflugers Arch. ges. Physiol.*, **232**, 299 (1933).
11. Feyel-Cabannes, T., *Compt. Rend. Ass. Anat.*, **51**, 178 (1947).
12. Glick, D., Techniques of histo- and cytochemistry. Interscience Publ., New York, 1949.
13. Hogeboom, G. H., Claude, A., and Hotchkiss, R. D., *J. Biol. Chem.*, **165**, 615 (1946).
14. Junqueria, L. C. U., Unpublished results (1948).
15. Junqueira, L. C. U., Fajer, A. B., Rabinovitch, M., and Frankenthal, L., *J. Cellular Comp. Physiol.*, **34**, 129 (1949).
16. Junqueira, L. C. U., Rabinovitch, M., and Fajer, A. B., *Rev. Brasil. Biol.*, **7**, 435 (1947).
17. Katagiri, S., and Higashijo, T., *Soc. Pathol. Jap. Trans.*, **30**, 252 (1940).
18. Keilin, D., *Proc. Roy. Soc. London B.*, **104**, 206 (1929).
19. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **172**, 847 (1948).
20. Kleczkowski, A., *Biochem. J.*, **42**, 523 (1948).
21. Kochakian, C. D., and Dontigny, P., *Proc. Soc. Exptl. Biol. Med.*, **67**, 61 (1948).
22. Korr, I. M. *in* Stotz, E., Cytochromes. A Symposium on respiratory enzymes, Wisconsin Press, Madison, p. 149, 1942.
23. Lacassagne, A., *Compt. Rend. Soc. Biol.*, **133**, 180 (1940).
24. Moog, F., *J. Cellular Comp. Physiol.*, **22**, 223 (1943).
25. Noback, C. R., and Montagna, W., *Am. J. Anat.*, **81**, 343 (1947).
26. Northup, D., *Am. J. Physiol.*, **114**, 46 (1935).
27. Pickford, G. E., and Dorris, F., *Science*, **80**, 317 (1934).
28. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **177**, 893 (1949).
29. Stannard, J. N., *Symposium Quant. Biol.*, Cold Spring Harbor, **7**, 394, (1939).
30. Stormont, D. L., *in* Cowdry's Special Cytology, P. B. Hoeber, N. York, **1**, 91 (1932).
31. Stowell, R. E., and Zorzoli, A., *Stain Technol.*, **22**, 51 (1947).
32. Tupa, A., *Bull. Histol.*, **3**, 293 (1926).

ALKALINE PHOSPHATASES IN FIXED PLANT CELLS

M. H. ROSS and J. O. ELY

Biochemical Research Foundation, Newark, Delaware

Received October 3, 1950

Alkaline phosphatase activity in plant cells has been demonstrated cytochemically for the first time. Reports of cytochemical findings on plant alkaline phosphatases presumably had been lacking because glycerophosphate, which is not hydrolyzed by fixed plant cells, had been used as the substrate. Studies on animal alkaline phosphatases heretofore had been confined chiefly to the demonstration of the site of enzyme activity on glycerophosphate.

Fixed plant tissues in this investigation were incubated also in substrate mixtures containing organic phosphate esters other than glycerophosphate. The enzymes in these tissues hydrolyzed some phosphate esters but did not hydrolyze others. Fixed animal tissues were incubated in the same phosphate esters and the enzymes hydrolyzed all of the organic phosphate esters employed.

MATERIALS AND METHODS

Plant tissues: Onion and corn root tips were chosen because of the size of the cells and great number of mitotic cells. Onions (*Allium cepa*) were germinated in tap water until the roots were 2–3 cm long. About 5 mm were cut from each root tip. These tips were fixed in 85 per cent ethyl alcohol for two hours with frequent changes of alcohol. Corn kernels (*Zea mays*) were germinated on filter paper moistened with tap water until the roots were about 6 cm long. These tips were also fixed in 85 per cent alcohol and were quickly dehydrated by passage through alcohol solutions of increasing concentrations, cleared in xylol and embedded separately in paraffin at 56–58° C. Longitudinal sections 6 microns in thickness were cut from blocks containing several of these roots and mounted. These tissue sections were then incubated at 40° C in phosphate substrates for 8, 16, 24, 48, or 72 hours. The tissue sections were then washed in distilled water, immersed in $Co(No_3)_2$ (2 per cent solution) for one min., washed again and immersed for two min. in a dilute solution of $(NH_4)_2$ S. After washing, the tissue sections were passed through a graded series of alcohols to xylol and mounted in Permount.

Animal tissues: Rat kidney, liver and duodenum were removed and fixed in 85 per cent ethyl alcohol. The tissues were dehydrated by passage through alcohol solutions of increasing concentrations, cleared in xylol and embedded in paraffin

blocks. Sections 6 microns in thickness were mounted on glass slides; egg albumen was used as the adhesive. After deparaffination and passage through alcohol-water mixtures down to water, the sections were incubated in different substrates for ½, 2, 4, 8, 16, 24 or 48 hours and subsequently treated in the same manner as the plant tissues.

Control tissues: Plant and animal tissues for comparison were subjected to the following:

1-Incubation in the buffered calcium-magnesium medium from which the phosphate-bearing substance was omitted;

2-Incubation at 80° C after deparaffination for various periods of time in order to heat-inactivate the enzyme;

3-Incubation in the substrate mixture in the presence of an "enzyme inhibitor";

4-Fixation of tissues in Carnoy fixative (acetic acid-ethyl alcohol, 1: 3).

Substrates employed:

Compound	Concentration Molar
Na β glycerophosphate (Eastman)	0.0026, 0.013, 0.05
Na glycerophosphate 52 per cent α, 48 per cent β (Eastman)	0.05
Disodium phenyl phosphate (E. and A.)	0.02
K diphenyl phosphate (prepared)	0.04
Na α napthyl acid phosphate (Dajac)	0.04
Ba phosphoglyceric acid (prepared)	0.03
Adenosine-3-phosphoric acid (Schwarz)	0.01, 0.02
Adenosine-5-phosphoric acid (Schwarz)	0.02
Adenosine diphosphate (Ba salt) (Syn-zyme)	0.01
Adenosine triphosphate (Schwarz)	0.004, 0.01
Diphosphopyridine nucleotide (Syn-zyme)	0.03
Guanosine-3-phosphoric acid (Schwarz)	0.02
Uridylic acid (Nutritional Biochem.)	0.025
Cytidylic acid (Nutritional Biochem.)	0.025
Ribosenucleic acid (yeast) (E. and A.)	approx. 0.02
Desoxyribosenucleic acid (calf thymus) (prepared)	» 0.006
Hypoxanthylic acid (prepared)	» 0.02
Xanthylic acid (prepared)	» 0.02
K glucose-1-phosphate (Nutritional Biochem. and Bios)	0.04
Na-d-glucose-6-phosphate (Fraenkel and Landau)	0.04
Ba fructose-6-phosphate (Schwarz)	0.04
Mg fructose-1, 6-phosphate (Schwarz, and prepared)	0.03
Ca fructose-1, 6-phosphate (Metz, and Bios)	0.02
Ba fructose-1, 6-phosphate (Nutritional Biochem.)	0.03
Na pyrophosphate (Merck)	0.002, 0.004, 0.03, 0.06
Na metaphosphate (Baker, and Fisher) 2 per cent colloidal suspension	0.0058
(filtrate)	0.04

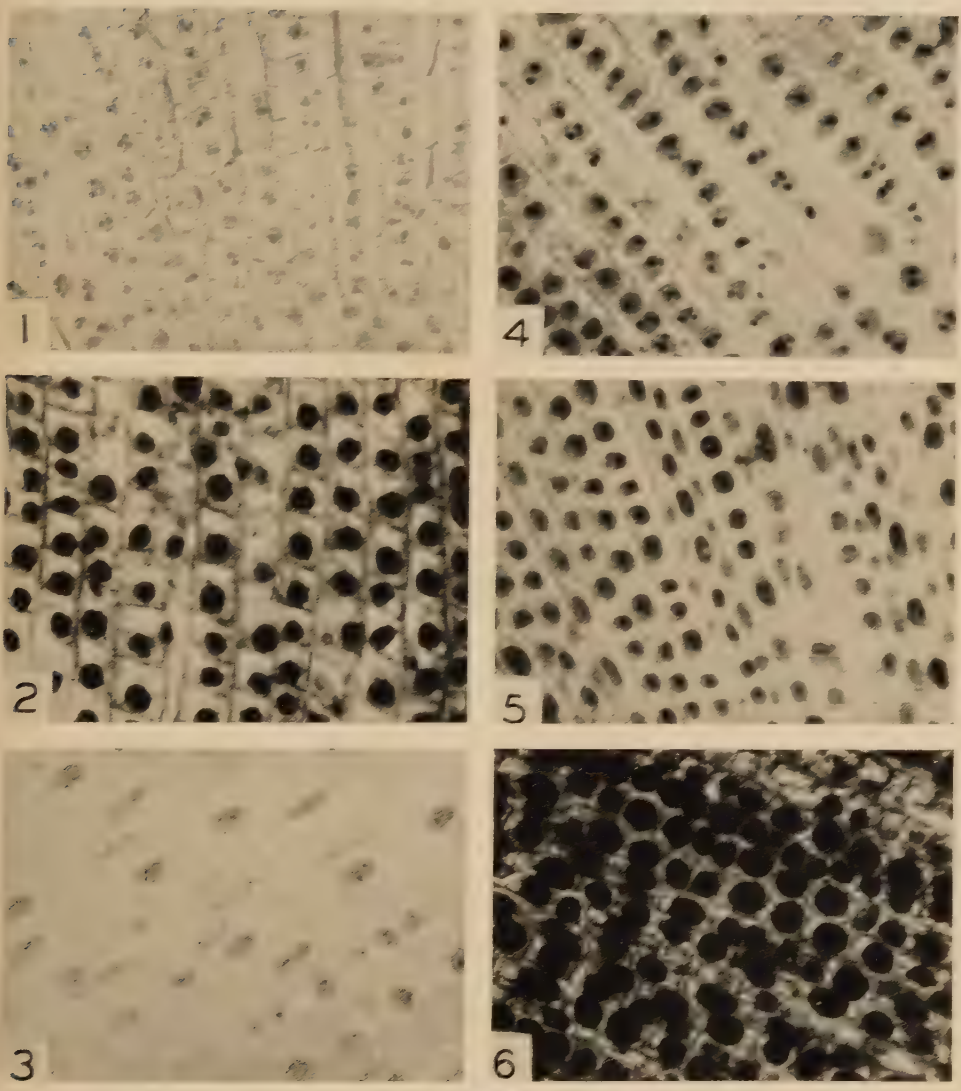


PLATE I

Substrate specificity of alkaline phosphatase in fixed onion root tip cells.

Fig. 1. Lack of activity in tissues incubated in 0.013 M Mg and β glycerophosphate for 48 hrs. Magnification = 400 X.

Fig. 2. Sites of activity in tissues incubated in 0.013 M adenosine triphosphate for 16 hrs. Magnification = 400 X.

Fig. 3. Lack of activity in tissues incubated in 0.02 M adenylic acid (yeast) for 24 hrs. Magnification = 400 X.

Figs. 4, 5, and 6. Sites of activity in tissues incubated in 0.02 M guanylic acid for 16, 24, and 48 hrs. respectively. Magnification = 400 X.

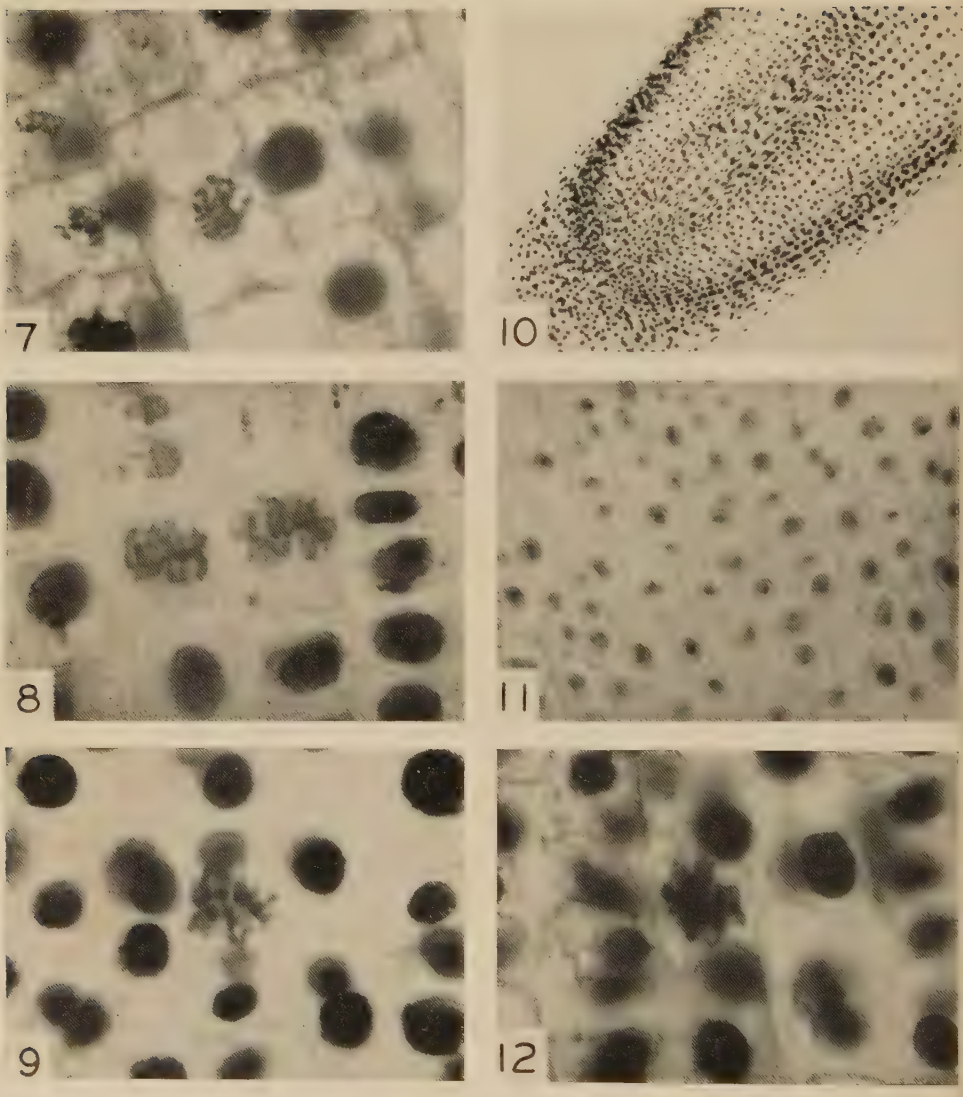


PLATE II

Sites of alkaline phosphatase activity in fixed onion root tip cells.

Figs. 7, 8 and 9. In tissues incubated in a 2 per cent suspension of Na metaphosphate. Chromosomes in prophase, metaphase and early anaphase respectively. Magnification = 938×.
Figs. 10, 11 and 12. In tissues incubated in 0.004 M Na pyrophosphate for 24, 24 and 48 hrs. respectively.
Magnification = 93×, 400× and 938× respectively.

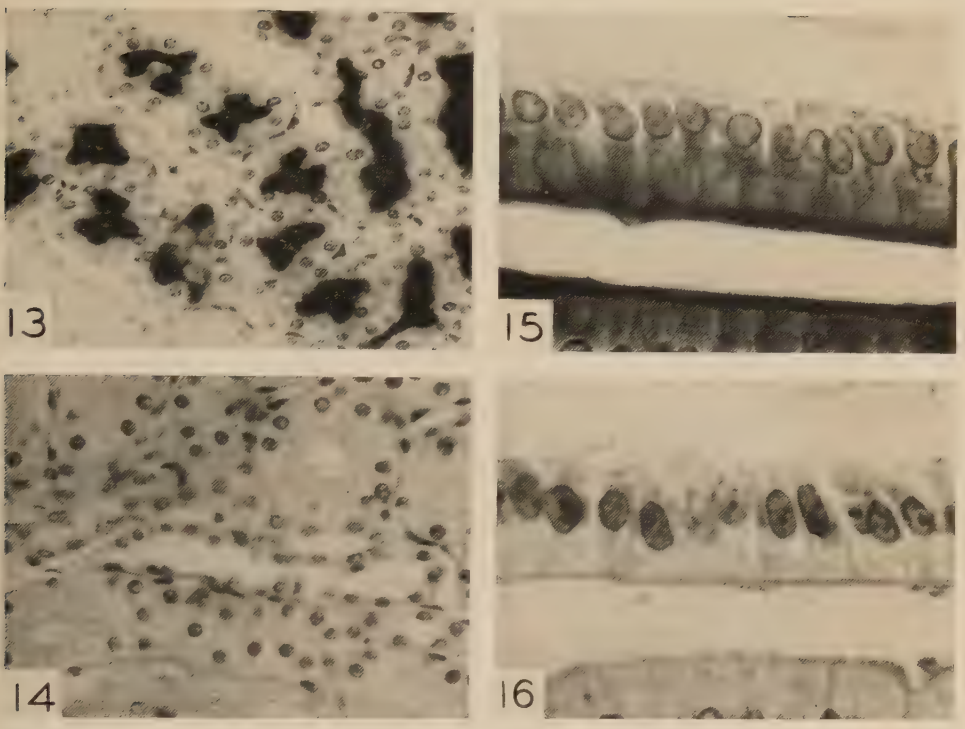


PLATE III

Sites of alkaline phosphatase activity in fixed rat kidney and duodenum cells.

Fig. 13. In proximal convoluted tubules. Tissues incubated in 0.0026 M Na glycerophosphate for 90 min. Magnification = 400 ×.

Fig. 14. In proximal convoluted tubules. Tissues incubated in 2 per cent suspension of Na metaphosphate for 44 hrs. Magnification = 400 ×.

Fig. 15. In villi. Tissues incubated in 0.0026 M Na glycerophosphate for 20 min. Magnification 938 ×.

Fig. 16. In villi. Tissues incubated in 2 per cent suspension of Na metaphosphate for 44 hrs. Magnification = 938 ×.

Hypoxanthylic acid and xanthylic acid were prepared from adenylic acid and guanylic acid respectively by Knopf's method described by Levene (6).

Polymerized desoxyribosenucleic acid was prepared from calf thymus by the method of Mirsky and Pollister (10) and was depolymerized by treatment with 1 N HCl at 60° C.

The substrates tested were made up according to the method of Gomori (3) with the glycerophosphate replaced by other phosphate compounds:

Phosphate compound	concentration given above.
$CaCl_2$	5 cc of a 2 per cent solution.
$MgSO_4$	2 cc of a 2 per cent solution.
Veronal buffer	10 cc of a 0.1 M solution.
Distilled water	enough to make 50 cc.

The pH of all substrate solutions was determined with the Beckman pH meter and adjusted to 9.4 with 1 N NaOH.

In some cases the suspension of Baker's Na metaphosphate was used, and in other cases the filtrate.

Enzymic activity: The black precipitate of CoS found within the cell was used as indication of enzymic activity, according to Gomori (3). It was assumed that the precipitate appeared only at the site of orthophosphate liberation, except for a slight diffusion (9), which may have occurred.

RESULTS

Hydrolysis in plant tissues: When alcohol-fixed tissues of onion and corn root tips were incubated in the substrates, enzyme hydrolysis of some, but not of other phosphate-bearing compounds was found to occur.

Substrates hydrolyzed	Substrates not hydrolyzed
Adenosine diphosphate	Na β glycerophosphate
Adenosine triphosphate	Na glycerophosphate (mixture of α and β salts)
Diphosphopyridine nucleotide	Na α napthyl acid phosphate
Guanylic acid	Adenosine-3-phosphoric acid
Ribosenucleic acid	Adenosine-5-phosphoric acid
Desoxyribosenucleic acid	Uridylic acid
Xanthylic acid	Cytidylic acid
Fructose-1,6-phosphate (Mg, Ca, and Ba salts)	Hypoxanthylic acid
Na metaphosphate	Disodium phenyl phosphate
Na pyrophosphate (freshly prepared solution)	K diphenyl phosphate
	Ba phosphoglyceric acid
	K glucose-1-phosphate
	Na-d-glucose-6-phosphate
	Ba fructose-6-phosphate

The order of appearance of phosphatase activity in plant cells for all organic substrates hydrolyzed was: in nucleoli, in nuclei, and in cytoplasm (see Figs. 4, 5, 6). Phosphatase activity was noted in the interphasic nuclei before it appeared in the mitotic figures (see Figs. 7, 8, 9).

The incubation time required was at least 18 hours in a 0.0058 M solution of metaphosphate (see Fig. 8) and at least 24 hours in 0.002 M pyrophosphate (see Figs. 10, 11) to obtain good cytological detail in the nuclei and nucleoli and 48 hours in the cytoplasm (see Fig. 12).

The intensity of the reaction was not appreciably affected by the addition

TABLE I

Phosphatase Activity Upon Addition of Fluoride and Magnesium Ions.

Substrate	NaF Added	In Plant Cells	In Animal Cells	$MgSO_4$ Added	In Plant Cells	In Animal Cells
Na glycerophosphate ..	0.1 M	none	none	0.0066 M	none	hydrolysis
	none	none	hydrolysis	none	none	hydrolysis
Guanylic acid	0.1 M	none	none	0.0066 M	hydrolysis	hydrolysis
	none	hydrolysis	hydrolysis	none	hydrolysis	hydrolysis
Adenosine triphosphate	0.1 M	none	none	0.0066 M	hydrolysis	hydrolysis
	none	hydrolysis	hydrolysis	none	hydrolysis	hydrolysis
Mg fructose-1, 6–PO_4..	0.1 M	none	none	0.0066 M	hydrolysis	hydrolysis
	none	hydrolysis	hydrolysis	none	hydrolysis	hydrolysis
Na metaphosphate	0.1 M	none	none	0.0066 M	hydrolysis	hydrolysis
	none	hydrolysis	hydrolysis	none	little	little
Na pyrophosphate	0.1 M	none	none	0.0066 M	hydrolysis	none
	none	hydrolysis	none	none	none	none

of an excess of $CaCl_2$ to the Na metaphosphate medium. The intensity of the reaction however was diminished by the addition of an excess of $CaCl_2$ to the Na pyrophosphate medium.

In the substrates which were not hydrolyzed, the nuclei were discerned as a faint grayish-brown mass and the nucleoli seen as a somewhat darker mass (see Figs. 1 and 3) which did not change on further incubation. The density of the precipitate was substantially the same as that found in the nuclei of the control tissues incubated for the same period of time in a mixture devoid of a phosphate substance.

Hydrolysis in animal tissues: When the alcohol-fixed rat tissues were incubated in the phosphate-bearing substrates, enzymic hydrolysis occurred with every organic phosphoester employed. Hydrolysis of inorganic Na metaphosphate also occurred but hydrolysis of Na pyrophosphate did not. (see Table I).

With all phosphoester substrates and in all three tissues examined, (duodenum, liver and kidney), the order of appearance of activity in the chromatin-bearing structures of the resting nucleus was the same as in plant cells. The site of alkaline phosphatase activity with glycerophosphate (see Figs. 13 and 15) was similar to that found with the other organic phosphoesters.

When the tissues were incubated in Na metaphosphate, intense activity

was found in the nucleoli and nuclei throughout all three tissues (see Figs. 14 and 16). It was not observed, however, in the brush borders of the proximal convoluted tubules of the kidney (see Fig. 14).

Control tissues: Effect of phosphotungstic acid. Control plant and animal tissues incubated in glycerophosphate, guanylic acid, adenylic acid, hexosediphosphate, adenosine triphosphate and metaphosphate substrates in the presence of 0.01 M phosphotungstic acid all failed to show phosphatase activity. The light gray-brown CoS precipitate, probably due to the adsorption of $Co(NO_3)_2$, was the same as that observed in tissues incubated in a mixture devoid of phosphates.

Effect of fluoride ions. The enzymic hydrolysis of 0.1 M glycerophosphate, guanylic acid, adenosine triphosphate, hexosediphosphates, Na metaphosphate and Na pyrophosphate was partially inhibited by a concentration of 0.01 M fluoride and completely inhibited by a concentration of 0.1 M fluoride (Table I).

Effect of magnesium ions. The effect of magnesium ions on alkaline phosphatase activity of plant and animal tissues on various substrates is shown in Table I.

DISCUSSION

Because guanylic acid and hexosediphosphates were hydrolyzed but adenylic acid and hexosemonophosphates were not it would appear that the enzymes present in fixed plant cells are not entirely nonspecific. In a previous study (11) on intestinal alkaline phosphatase activity it was suggested that it is one and the same enzyme which is responsible for the hydrolysis of desoxyribosenucleic acid, glycerophosphate and adenylic acid. The results reported here indicate that more than one enzyme may be responsible for the hydrolysis of the substrates employed in the present study.

Since it may be assumed that in the living plant cell dephosphorylation of all phosphoric acid esters occurs, the lack of hydrolysis of some of these substances in the fixed plant cell does not necessarily indicate a lack of the enzyme in the living cell but rather the presence of another enzyme whose activity is not demonstrable by the present cytological methods. In plant tissues, fixation and embedding may cause the inactivation of these particular enzymes or may even cause the loss of these enzymes by diffusion. In animal tissues, on the other hand, fixation and embedding does not appear to produce this effect; the enzyme may be more resistant to inactivation or may be bound more tightly to other components of the cell. Or, it may be that in animal cells the enzymes may differ from those in plant

cells. It may well be that a loss of the particular enzyme which hydrolyzes glycerophosphate occurred in the cytological preparations of tissues which have been reported (1, 15, 16, 17) to be lacking in alkaline phosphatase activity. For example, Yao has reported (18) that alkaline phosphatase activity is not present in *Drosophila* ovaries and testes and during the early developmental period of the embryo.

The fact that hydrolysis was obtained with guanylic acid but not with either yeast or muscle adenylic acid cannot be explained by the configuration of the sugar molecule (2) or by the position of the phosphate linkage. The hydroxyl group of the purine base of hypoxanthylic acid, prepared from adenylic acid, corresponds to the hydroxyl group of the purine base of guanylic acid. Hypoxanthylic acid like adenylic acid was found not to be hydrolyzed. Xanthylic acid prepared from guanylic acid was hydrolyzed. Apparently an amino group or a hydroxyl group in the 2 position may be necessary for phosphatase activity on nucleotides.

Since inorganic pyrophosphate was acted upon by an enzyme in plant tissues and since there is a pyrophosphate linkage in adenosine diphosphate and adenosine triphosphate, it may be that these substances were hydrolyzed by an enzyme which acts on the pyrophosphate bond. Since adenylic acid was not hydrolyzed by the enzymes in the onion root, the inorganic phosphate may have been liberated from diphosphopyridine nucleotide by an initial enzymic hydrolysis of the pyrophosphate (5) followed by hydrolysis of the nicotamide-ribose-phosphate. Because adenylic acid and adenosine diphosphate were hydrolyzed by animal tissues it is probable that both phosphate groups of the diphosphopyridine nucleotide molecule were liberated.

The enzyme in the nuclei of plant and animal tissues which was found to convert metaphosphates to orthosphates may be similar to the metaphosphatase in *Aspergillus niger* reported by Mann (7). The metaphosphates which had been found in a few microorganisms (4, 7, 8, 13, 14) may be the form in which phosphates are stored for future conversion into orthophosphates. The localization of the metaphosphatase within the nucleus may be considered as support for Schlenk's suggestion (12) that metaphosphates may supply orthophosphates for nucleic acid synthesis.

SUMMARY

Alkaline phosphatase activity in fixed plant cells has now been demonstrated cytochemically. Presumably cytochemical findings on plant alkaline

phosphatases had been lacking because glycerophosphate, which is not hydrolyzed by fixed plant cells, had been used as the substrate.

Alkaline phosphatase activity in the onion and corn nuclei has been compared with the activity in rat tissues. In the plant tissues, hydrolysis of phosphates was demonstrated when the substrates guanylic acid, adenosine diphosphate, adenosine triphosphoric acid, diphosphopyridine nucleotide, hexosediphosphates and inorganic pyrophosphate and metaphosphate were used. When the substrates glycerophosphate, adenylic acid and hexosemonophosphates were used, hydrolysis was not found. In the animal tissues however, hydrolysis was demonstrated of all organic phosphoesters employed and of sodium metaphosphate but not the hydrolysis of sodium pyrophosphate.

One alkaline phosphatase found in the fixed plant tissues specifically hydrolyzed guanylic acid but no other nucleotide and one specifically hydrolyzed metaphosphate to orthophosphate.

The enzymes in both plant and animal cells which hydrolyzed metaphosphates and pyrophosphates were found to require magnesium ions for their activity and to be inhibited by fluoride ions.

"Alkaline phosphatase," so intimately associated with the chromatin in the nucleus, is postulated to be not just one enzyme but a number of enzymes.

REFERENCES

1. Bullock, W. L., *J. Morphol.*, **84**, 185 (1949).
2. Davoll, J., Lythogoe, B., and Todd, A. R., *J. Chem. Soc.*, 833 (1946).
3. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **42**, 23 (1939).
4. Ingelman, B., *Svensk Kemisk Tidskrift*, **60**, 222 (1948).
5. Kornberg, A., and Lindberg, O., *J. Biol. Chem.*, **175**, 665 (1948).
6. Levene, P. A., and Bass, L. W., Nucleic Acids, New York, 1931.
7. Macfarlane, M. G., *Biochem. J.*, **30**, 1369 (1936).
8. Mann, T., *Biochem. J.*, **38**, 339 and 345 (1944).
9. Martin, B. F., and Jacoby, F., *J. Anat.*, **83**, 351 (1949).
10. Mirsky, A. T., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 117 (1946).
11. Ross, M. H., and Ely, J. O., *J. Cellular Comp. Physiol.*, **34**, 71 (1949).
12. Schlenk, F., *Advances in Enzymol.* **9** (1949).
13. Schmidt, G., Hecht, L., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 775 (1946).
14. Sommer, A. L., and Booth, T. E., *Plant Physiol.*, **13**, 199 (1938).
15. Wang, K. J., and Grossman, M. I., *Anat. Record*, **104**, 79 (1949).
16. Weisz, P. B., *Biol. Bull.*, **97**, 108 (1949).
17. Willmer, E. N., *J. Exptl. Biol.*, **19**, 11 (1942).
18. Yao, T., *Quart. J. Microscop. Sci.*, **91**, 79 (1950).

THE INHIBITION OF PANCREAS DEOXYRIBONUCLEASE

LISELOTTE M. GILBERT, W. G. OVEREND and M. WEBB

Department of Chemistry, The University, Birmingham and Strangeways Research Laboratory, Cambridge, Great Britain

Received October 10, 1950

Recent communications (30, 31) have described the results of an investigation of the properties and mode of action of deoxyribonuclease isolated from beef-pancreas by essentially the method of McCarty (28). These and additional studies have shown that the action of this enzyme, which requires magnesium ions for its activation, is analogous to that of the corresponding ribonuclease (32) but is confined specifically to the deoxyribo type of nucleic acid or its partially degraded products (16).

In view of the increased use of deoxyribonuclease in cytochemical work (8, 24), together with the possible significance of enzymes of this type in the processes of cell division, a detailed investigation of the inhibition of the enzyme appeared justified. Certain preliminary observations have been mentioned briefly in a previous communication (31), and the present paper describes fully the extension of this work. In addition, a study has been made of the action of deoxyribonuclease on the deoxyribonucleohistone of calf thymus cell nuclei alone and in the presence of substances known to inhibit mitosis.

MATERIAL AND METHODS

Deoxyribonuclease was isolated from beef-pancreas by McCarty's (28) method, and was purified by repeated fractional precipitation with ammonium sulphate (31).

Deoxyribonucleic acid. Highly polymeric preparations of deoxyribonucleic acid were isolated from calf-thymus and herring sperm according to the method of Mirsky and Pollister (29). The chemical and physical properties of these preparations N/P (= = 1.42 & 1.56 respectively) will be described elsewhere. In addition to these preparations, commercial (B. D. H.) deoxyribonucleic acid was also used as substrate. After purification as previously described (31) the nucleic acid had N/P : 1.6.

Deoxyribonucleohistone was isolated with 0.002 *M* sodium arsenate (33) from the gelatinous mass of thymus cell nuclei remaining after the extraction of the minced tissue with 0.14 *M* sodium chloride. The nucleoprotein was precipitated by the addition of sodium chloride to 0.14 *M* concentration, and purified by repeated solution in 0.002 *M* sodium arsenate and precipitation with sodium chloride. Finally, it was dissolved in distilled water, precipitated with cold ethanol (1 vol.) and dried with

ethanol and ether. The residual white powder (N, 15.6; P, 5.05 per cent), which contained a negligible amount of arsenate (Marsh test and potassium iodide reaction), dissolved readily in water to give a viscous, opalescent solution. The product gave a positive reaction for sulphur after sodium fusion, but the nitroprusside reaction for -SH groups was negative before, and only weakly positive after, treatment with potassium cyanide (22).

EXPERIMENTAL

The inhibition of the action of Deoxyribonuclease on Thymus Deoxyribonucleic acid

The inhibitory action of various substances on the activity of deoxyribonuclease was determined initially by comparative measurements of the formation of "acid-soluble" polynucleotide material as follows: —

Method I. A series of test-tubes each containing 2 per cent ($^{w}/_{v}$) purified commercial thymus sodium deoxyribonucleate (2.0 ml), 0.1 *M* magnesium sulphate (1.0 ml), 0.1 *M* veronal buffer pH 7.0 (1.0 ml), 0.01 per cent ($^{w}/_{v}$) deoxyribonuclease (0.5 ml) and the solution (0.5 ml) of the substance under examination, was incubated at 37°. After 16 hrs the tubes were removed from the incubator, cooled to 0° and aliquots (4.0 ml) of the contents transferred to tared centrifuge tubes. 5 *N* hydrochloric acid (0.1 ml) was then added to each and the resulting "acid insoluble" precipitate collected at the centrifuge. The deposit was washed at the centrifuge with ethanol, ethanol and ether and finally with ether. After standing overnight *in vacuo* at room temperature, the tubes were re-weighed and the amount of "acid insoluble" material determined by difference. Each determination was carried out in triplicate. By this method the following substances were examined for inhibitory action:—0.1 *M* sodium arsenate, 0.1 *M* sodium selenite, 0.5 *M* thioglycollic acid neutralised to pH 8.0, 0.3 *M* potassium fluoride (in the presence of 0.2 *M* phosphate buffer pH 7.9), 0.1 *M* hydroxylamine, 0.1 *M* cysteine, and 0.03 *M* iodoacetic acid.

The results obtained (Table I) show that the enzyme was almost completely inhibited by arsenate, selenite and fluoride ions, and partially inhibited by thioglycollate.

Method II. Since the use of the above method was only suitable in cases where the inhibitory substances remained in solution in the presence of dilute acid, the following procedure proved of more general application:—

A series of test-tubes, each containing 1.0 per cent ($^{w}/_{v}$) purified sodium deoxyribonucleate (2.0 ml), 0.1 *M* magnesium sulphate (1.0 ml), 0.01 per cent ($^{w}/_{v}$) deoxyribonuclease (1.0 ml) and a solution (1.0 ml) of the inhibitory

TABLE I

The inhibition of deoxyribonuclease by various substances under the conditions described in Method I (see Text).

Inhibitor	Percentage of deoxyribonucleic acid converted to acid soluble material in 16 hours at 37°
0.1 *M* Sodium selenite	c. 5
0.1 *M* Sodium arsenate	c. 5
0.5 *M* Thioglycollic acid	44
0.3 *M* Potassium fluoride	10
0.1 *M* Hydroxylamine	95
0.1 *M* Cysteine	94
0.03 *M* Iodoacetic acid	91
Control (No Inhibitor)	95

substance, was incubated at 37°. At suitable time intervals (Fig. 1) the contents of one tube were treated with 5 *N* hydrochloric acid (0.1 ml) in order to precipitate the residual "acid-insoluble" material. After centrifuging, the extent of the hydrolysis, in terms of the increase in acid soluble phosphorus, was determined by Allen's (1) method in an aliquot of either the clear supernatant, or a solution of the "acid insoluble" precipitate in 0.05 *M* sodium carbonate.

The following substances were examined for inhibitory action by this method.—

(a) 0.01 *M* copper sulphate; (b) 0.1 *M* zinc sulphate; (c) 0.01 *M* zinc sulphate; (d) 0.125 *M* sodium sulphide; (e) 0.1 *M* potassium fluoride; (f) 0.01 *M* potassium fluoride; (g) 0.1 *M* sodium citrate adjusted to pH 7.0; (h) 0.01 *M* sodium citrate adjusted to pH 7.0; (i) 0.1 *M* sodium borate; (j) 0.01 *M* hydroxylamine hydrochloride; (k) 0.01 *M* semicarbazide hydrochloride; (l) 0.01 *M* sodium bisulphite; (m) 6×10^{-4} *M* iodoacetic acid; (n) 6×10^{-4} *M* iodoacetamide.

Of these substances a, b, c, d, e, g and i completely inhibited, and substances f and h partially inhibited the action of the enzyme (Fig. 1).

The inhibitory action of fluoride and citrate ions on the activity of systems containing deoxyribonuclease and magnesium was reported by McCarty (28), whilst the inhibition of the enzyme by arsenate was noted by Fischer, Bottger and Lehmann-Echternacht (14). Since dilute sodium arsenate (or sodium citrate) has been proposed as a solvent for the isolation of undegraded deoxyribonucleoproteins (33), it was of interest to determine the

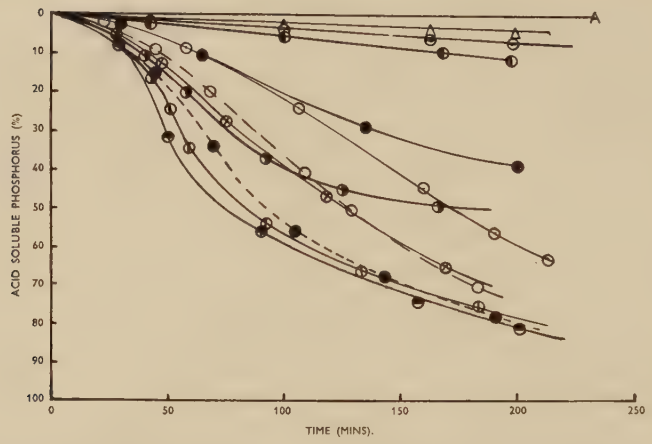


Fig. 1. *Effect of various substances upon the hydrolysis of thymus deoxyribonucleic acid by deoxyribonuclease.* Conditions as described in Method II.
◓——◓ control (no inhibitor), △——△ 0.1 *M* zinc sulphate, ⊖——⊖ 0.01 *M* zinc sulphate, ◐——◐ 0.1 *M* sodium citrate, •——• 0.01 *M* sodium citrate, ○——○ 0.01 *M* semicarbazide hydrochloride, ◑——◑ 0.01 *M* potassium fluoride, ○ — — ○ 0.01 *M* hydroxylamine hydrochloride, ⊗——⊗ 0.01 *M* sodium bisulphite, • - - - - • 6×10^{-4} *M* iodoacetic acid. ⊕——⊕ 6×10^{-4} *M* iodoacetic acid. Curve *A*, 0.01 *M* copper sulphate, 0.125 *M* sodium sulphide, 0.1 *M* potassium fluoride and 0.1 *M* sodium borate.

relationship between the concentration of arsenate and the degree of inhibition, in systems containing constant amounts of nucleic acid, magnesium, and enzyme.

Effect of concentration of sodium arsenate on the activity of deoxyribonuclease

A series of tubes containing a buffered solution of deoxyribonucleic acid, deoxyribonuclease, Mg^{++} and varying concentrations of sodium arsenate (10^{-1} to 10^{-6} *M*) was prepared according to Method I (above), and incubated at 37°. After 16 hours, the "acid-soluble" material remaining in each tube was determined as previously described. The results (Fig. 2) show that under the conditions of the experiment a final concentration of 1×10^{-3} *M* sodium arsenate was required to inhibit the enzyme completely.

It has been shown (30, 31) that the action of deoxyribonuclease on highly polymerised preparations of deoxyribonucleic acid results initially in a rapid depolymerisation of the substrate as evidenced by a striking decrease in the relative viscosity of the solution. During this period the products which result from the action of the enzyme are completely insoluble in dilute acids, and only when the relative viscosity has reached a constant

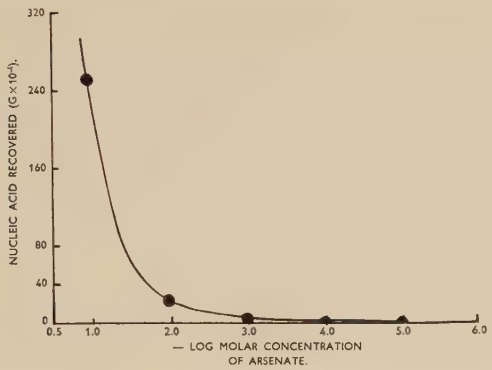


Fig. 2. *The effect of varying concentrations of sodium arsenate on the activity of deoxyribonuclease.* Measurements of acid insoluble material (recovered nucleic acid) were made after 16 hours at 37° according to Method I.

value does the liberation of acid-soluble material occur to any marked extent. From then onwards, the formation of products soluble in dilute mineral acids follows a course typical of enzymic hydrolysis. The action of the enzyme on various commercial samples of deoxyribonucleic acid follows a similar course, but because these preparations are already partially degraded, the initial change in relative viscosity occurs between narrower limits.

Recently Jungner, Jungner and Allgén (25) and Vercauteren (34) independently have studied the enzymic degradation of deoxyribonucleic acid by different techniques with closely similar results, whereas Greenstein, Carter and Chalkley (19) conclude that the initial disaggregation of the nucleic acid molecule, as shown by the changes in relative viscosity, may be induced by a variety of inorganic and organic electrolytes, and is not due specifically to the action of the enzyme.

In consequence of the mode of action of deoxyribonuclease, as outlined above, and in particular, of the conclusions of Greenstein *et al.* (19), it was of interest to determine whether the substances previously shown to inhibit the overall action of the enzyme, inhibited both the depolymerisation and the hydrolysis of the substrate.

Influence of inhibitors on the depolymerisation of deoxyribonucleic acid

The enzymic depolymerisation of the deoxyribonucleic acid was followed viscosimetrically at 38.5°. Measurements were made either in Ostwald viscometers or in viscometers of the type described by Koch, Orthmann and

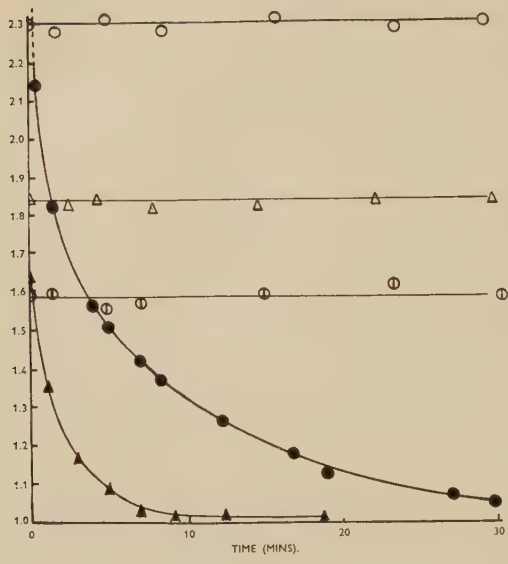


Fig. 3 A.

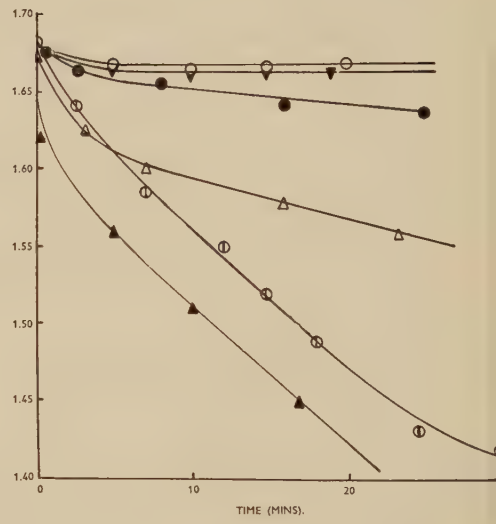


Fig. 3 B.

Fig. 3 A. *The action of inhibitors on the depolymerisation of calf thymus and herring sperm deoxyribonucleic acids.* The relative viscosities (η_r) of the solutions are plotted as the ordinate, and are taken as a measure of the polymerisation of the substrate. All solutions contained the deoxyribonucleic acid (0.2 per cent, 2 ml), 0.2 *M* veronal buffer pH 6.92 (1.0 ml), 0.1 *M* magnesium sulphate, deoxyribonuclease (0.002 per cent, 0.5 ml) and the inhibitor (0.5 ml).

▲——▲ Herring sperm deoxyribonucleic acid substrate, no inhibitor.
⦶——⦶ Herring sperm deoxyribonucleic acid substrate. 0.01 *M* zinc sulphate.
●——● Thymus deoxyribonucleic acid substrate. No inhibitor.
△——△ Thymus deoxyribonucleic acid substrate. 0.01 *M* sodium arsenate.
○——○ Thymus deoxyribonucleic acid substrate. 0.01 *M* sodium selenite.

Fig. 3 B. *The action of inhibitors on the depolymerisation of purified (commercial) thymus deoxyribonucleic acid.* All solutions contained the deoxyribonucleic acid solution (8 per cent, 2 ml), 0.2 *M* veronal buffer pH 6.92 (1.0 ml), 0.1 *M* magnesium sulphate (1.0 ml), deoxyribonuclease (0.002 per cent, 0.05 ml) and the inhibitor (0.5 ml).
▲——▲ control (no inhibitor), ⦶——⦶ 0.01 *M* semicarbazide hydrochloride, △——△ 0.01 *M* copper sulphate, ●——● 0.01 *M* sodium arsenate, ▼——▼ 0.01 *M* zinc sulphate, ○——○ 0.01 *M* sodium citrate.

Degenfelder (26). Both the highly polymerised preparations of the deoxyribonucleic acids from thymus and herring sperm (0.2 per cent ($^{w}/_{v}$) solutions) and the purified commercial deoxyribonucleic acid (8.0 per cent ($^{w}/_{v}$) solution) were used in these experiments. The buffered substrate mixture containing the deoxyribonucleic acid solution (2.0 ml), 0.2 *M* veronal buffer, pH 6.92 (1.0 ml), 0.1 *M* magnesium sulphate (1.0 ml) and the inhibitor (0.5 ml), was introduced into the viscometer, and the temperature allowed to equilibrate. The deoxyribonuclease solution (0.002 per cent ($^{w}/_{v}$), 0.5 ml, preheated

to 38.5°) was then added and viscosity measurements commenced immediately after mixing. The following substances were examined for inhibitory action on the depolymerisation under the above conditions: sodium arsenate, sodium selenite, zinc sulphate, copper sulphate, sodium citrate (adjusted to pH 7.0), semicarbazide hydrochloride and hydroxylamine hydrochloride (Figs. 3 A and 3 B). The fact that arsenate, selenite, citrate, copper and zinc ions inhibit the enzymic depolymerisation of deoxyribonucleic acid, proves that the change in viscosity observed in the control experiments (without inhibitor) is due specifically to the action of deoxyribonuclease, and not to the non-specific action of metallic ions (19).

Influence of inhibitors on the hydrolysis of depolymerised deoxyribonucleic acid

The experiments outlined under Method II above, were repeated with the exception that the addition of substances (a) to (n) was not made until 20 minutes after the addition of the enzyme (i.e. until depolymerisation was more or less complete). From then on the liberation of acid soluble polynucleotide material was followed by phosphorus estimations in the usual way. Under these conditions the action of the enzyme was arrested by those substances which inhibited the depolymerisation of the deoxyribonucleic acid (Fig. 3).

The Hydrolysis of Deoxyribonucleohistone by Deoxyribonuclease

The action of deoxyribonuclease on the deoxyribonucleohistone from calf thymus nuclei was studied viscosimetrically in Ostwald viscometers (flow time for water (4.5 ml) 85–90 secs.) at 34.5° ± 0.25°. The deoxyribonucleohistone was dissolved in water by stirring mechanically, and the resulting opalescent, viscous solution filtered through a plug of cotton wool. The filtrate was suitably diluted with water until a test sample (2.0 ml) together with 0.1 *M* veronal buffer pH 6.9 (1.0 ml) and distilled water (1.5 ml) had a flow time in the viscometers of 3 to 4 minutes. The concentration of the nucleoprotein solution (0.9 per cent $^{w}/_{v}$) was then determined by phosphorus analyses and by drying and weighing the precipitate produced by the addition of ethanol (2 vols) to an aliquot (10 ml) of the solution.

A gradual decrease in the relative viscosity occurred with time in mixtures of the nucleoprotein (2.0 ml), veronal buffer (1.0 ml) and distilled water (1.5 ml), (Fig. 4). Since this change was not observed when the nu-

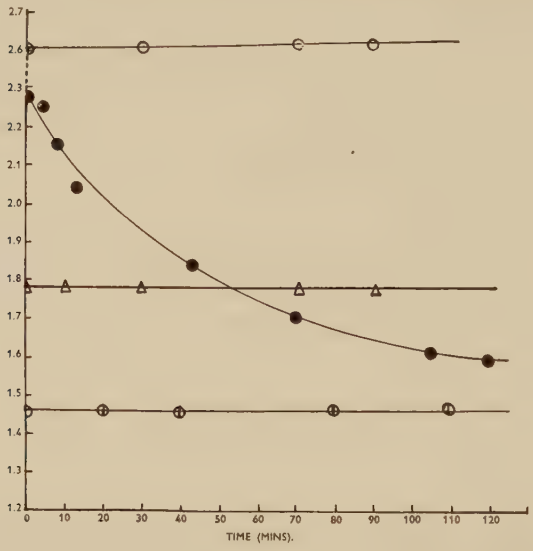


Fig. 4. Self hydrolysis (depolymerisation) of thymus deoxyribonucleohistone at pH 6.9 (● —— ●) and its inhibition by 2 *M* sodium chloride (○——○), 0.01 *M* sodium arsenate (⦶——⦶) and 0.1 *M* sodium fluoride (△——△).

cleoprotein solution (2.0 ml) was diluted with 2.0 *M* sodium chloride (2.0 ml), 0.05 *M* sodium arsenate (2.0 ml) or 0.05 *M* sodium fluoride (2.0 ml), (Fig. 4), it is concluded that a small amount of thymus deoxyribonuclease (37) was associated with the nucleoprotein.

In initial experiments it was observed that the addition of magnesium ions (essential for the action of deoxyribonuclease with deoxyribonucleic acid as substrate) to the deoxyribonucleohistone solution, resulted in the precipitation of the nucleoprotein. Qualitative experiments in which the nucleoprotein solution (1.0 ml) was added to 0.2 per cent ($^{w}/_{v}$) solutions of various metallic salts, showed that precipitation occurred with all the metallic ions examined with the exception of sodium and potassium. From a visual estimation it was possible to arrange the metallic ions in the following order according to the bulk of precipitate produced and the rate at which it separated:—

$$Ag^{+} > Hg^{++} > Fe^{+++} > Be^{++} > Pb^{++} > Cu^{++} > Ba^{++} = Ca^{++} = \\ = Mn^{++} > Fe^{++} > Mg^{++}.$$

The effect was limited to cations, and was not a non-specific effect due to ionic concentration, since the precipitates failed to dissolve when diluted with water (10 vols.). Furthermore, it was not a peculiar property of the

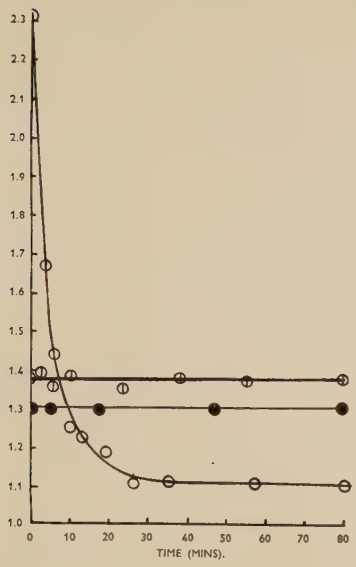


Fig. 5.

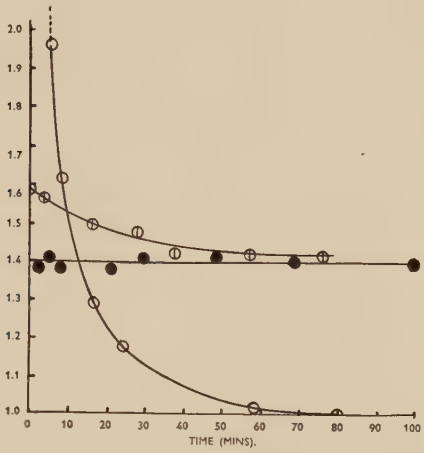


Fig. 6.

Fig. 5. The action of deoxyribonuclease on thymus deoxyribonucleohistone (○——○) and its inhibition by the addition of 1.0 ml 0.1 *M* sodium fluoride (•——•) or 0.1 *M* sodium citrate (◑——◑). Hydrolysis of the substrate shown by the decrease in relative viscosity (η_r). Each solution contained the deoxyribonucleohistone solution (2.0 ml), 0.2 *M* veronal buffer pH 6.9 (1.2 ml) and 0.002 deoxyribonuclease (0.3 ml).

Fig. 6. The action of 0.1 *M* sodium arsenate (◑——◑), 0.1 *M* sodium selenite (•——•) and 0.1 *M* hydroxylamine or cysteine (○——○) on the hydrolysis of deoxyribonucleohistone by deoxyribonuclease. Conditions were as in Fig. 5. Hydroxylamine and cysteine produced no inhibition of the enzyme. Thus, the curve (○——○) coincides with that showing the action of the enzyme alone on thymus deoxyribonucleohistone.

deoxyribonucleohistone isolated by Stern's (33) method, since the same effect was observed when solutions of fibrous nucleoprotein, isolated from thymus with 1.0 *M* sodium chloride according to the method of Mirsky and Pollister (29), were treated with the metallic ions listed above. In the few cases examined, precipitation of the nucleoprotein appeared to be complete, since the clear solutions obtained after removing the insoluble (Ag^+ Be^{++} and Mg^{++}) "salts" (centrifuge) gave negative reactions for deoxyribose with the Dische (9) dephenylamine reagent.

It was observed that the Ca^{++}, Fe^{++}, Be^{++} and Mg^{++} "salts" of the nucleohistone were completely soluble in 2 *M* sodium chloride. In this solvent, the Cu^{++} and Ba^{++} "salts" were partially soluble and yielded suspensions of high viscosity, whereas the Ag^+, Hg^{++} and Fe^{+++} "salts" remained undissolved.

Since the precipitating effect of Mg^{++} and other metallic ions on thymus nucleohistone rendered it impossible to follow viscosimetrically the action of magnesium-activated deoxyribonuclease on this substrate, the action of the enzyme on the nucleoprotein was studied in the absence of free magnesium ions. Under these conditions the enzyme exhibited high activity and caused the rapid hydrolysis of the substrate (Fig. 5). Since this hydrolysis was inhibited when either 0.1 *M* sodium fluoride (1.0 ml) or 0.1 *M* sodium citrate (1.0 ml) was added to solutions containing deoxyribonucleohistone (2.0 ml), 0.2 *M* veronal buffer (1.2 ml) and 0.002 per cent ($^{w}/_{v}$) deoxyribonuclease (0.3 ml), (Fig. 5), it appeared that the enzyme-substrate system was activated by magnesium present in the nucleohistone. In this connection the nucleoprotein yielded 10.55 per cent ash on combustion which, when analysed according to the method previously described (35), was found to contain 0.15 per cent magnesium (≡ a magnesium content of 0.016 per cent in the nucleohistone).

In addition to sodium citrate and sodium fluoride, 0.1 *M* sodium arsenate (1.0 ml) and 0.1 *M* sodium selenite (1.0 ml) were found to inhibit the action of deoxyribonuclease (0.002 per cent, 0.3 ml) on deoxyribonucleohistone (2.0 ml) in 0.1 *M* veronal buffer pH 6.92 (1.2 ml), (Fig. 6), whereas equivalent concentrations of hydroxylamine hydrochloride (in 0.1 *M* sodium acetate) and cysteine were without effect upon the activity of the enzyme (Fig. 6). It will be noted from these results (Fig. 6) that the increased ionic strength, due to the presence of the "inhibitors," reduced the initial relative viscosity of the nucleoprotein solution.

The influence of mitotic inhibitors on the hydrolysis of deoxyribonucleohistone by deoxyribonuclease

It appeared possible that certain inhibitors of mitosis exert their action by reacting with nuclear constituents and thereby preventing subsequent enzymic processes. It was of some interest, therefore, to determine whether such substances exerted any effect upon the degradation of the nuclear deoxyribonucleohistone by deoxyribonuclease. Particular attention was directed to the -SH reactants chloroacetophenone, iodoacetic acid and iodoacetamide (Group I) which cause telophase inhibition in cells in chick tissue cultures (Hughes 23). In addition, the action of colchicine and aminopterin (4-amino-pteroylglutamic acid) (Group 2) and certain nitrogen mustards (Group 3) was studied.

For the evaluation of the action of compounds of groups 1 and 2, the experimental technique was as follows:—

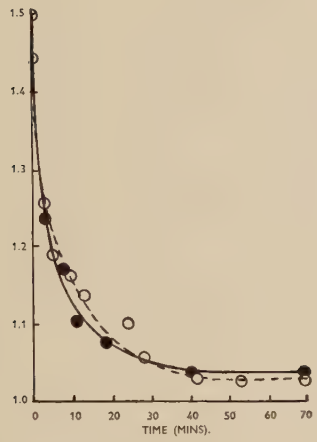


Fig. 7.

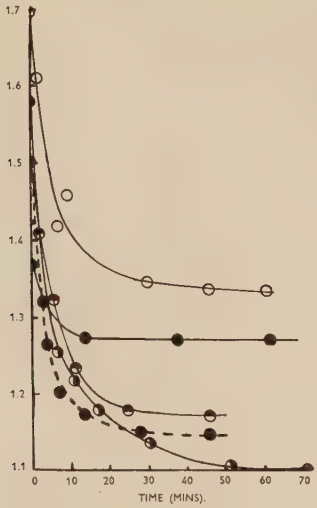


Fig. 8.

Fig. 7. The hydrolysis of deoxyribonucleohistone with deoxyribonuclease alone (• —— •) and in the presence of 1.0 ml 0.01 *M* iodoacetamide (○ - - - - ○) under the conditions recorded in the text. The curves showing the hydrolysis of the substrate with time in the presence of aminopterin (1 mg) and chloroacetophenone (1 ml of a saturated aqueous solution) coincide with the above, and are not recorded in the figure.

Fig. 8. *The action of deoxyribonuclease on thymus deoxyribonucleohistone after treatment with nitrogen mustards.*
◑——◑ control-deoxyribonucleohistone with deoxyribonuclease as in Fig. 5. Deoxyribonucleohistone treated directly with (1) N.N. di-(2-chloroethyl)-p-amino benzoic acid (○ ——○) and (2) β-naphthyl-di-(2-chloroprophyl)amine (• —— •) according to method (a) (p. 359). The curves (◓——◓) and (• - - - - •) show the action of deoxyribonuclease on the deoxyribonucleohistone after treatment with buffered aqueous solutions of β-naphthyl-di-(2-chloropropyl)amine and N.N. di-(2-chloroethyl)-p-aminobenzoic acid respectively, which had been kept at room temperature for 20 mins.

A mixture of the nucleoprotein solution (2.0 ml) 0.1 *M* veronal buffer pH 6.92 (1.2 ml) and the inhibitor solution (1.0 ml) was allowed to stand at room temperature for 20–60 minutes. It was then introduced into the viscometer, the temperature allowed to equilibrate, and deoxyribonuclease (0.002 per cent ($^{w}/_{v}$) 0.3 ml) added. Measurements of relative viscosity were made at frequent intervals during the following 30–60 minutes.

The action of compounds of group 3 was determined according to the following methods:—

(a) To the mechanically stirred nucleoprotein solution (8.0 ml) was added a solution of the nitrogen mustard (8.0 mg) in the minimum volume of ethanol necessary for complete solution. The resulting turbid solution was stirred for a further 15 minutes and then allowed to stand at room temper-

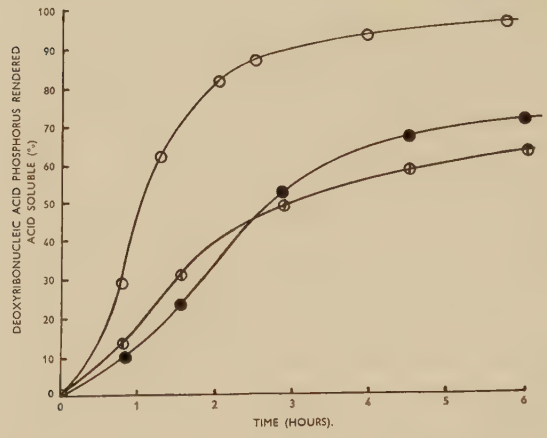


Fig. 9.

Fig. 9. *The hydrolysis by deoxyribonuclease of thymus deoxyribonucleohistone treated with nitrogen mustards.* Hydrolysis measured by the liberation of acid soluble phosphorus, expressed as a percentage of the total phosphorus of the substrate, according to method (c) (p. 360). ○——○. The action of deoxyribonuclease on thymus deoxyribonucleohistone alone (control). ⓘ——ⓘ and •——•, the action of deoxyribonuclease on deoxyribonucleohistone treated with N.N. di-(2-chloroethyl)-p-aminobenzoic acid and β-naphthyl-di-(2-chloropropyl)-amine respectively.

ature for 60 minutes, (marked precipitation occurred in such solutions on prolonged standing). At the end of this period an aliquot of the solution was diluted with 0.1 *M* veronal buffer pH 6.9 (2.0 ml) and then introduced into the viscometer. After equilibration of temperature, deoxyribonuclease (0.002 per cent, 0.5 ml) was added and viscosity measurements commenced.

(b) The ethanolic solution of the nitrogen mustard (4 mg) was added with stirring to 0.1 *M* veronal buffer pH 6.9 (4.0 ml). This was followed after 20 minutes by the addition of the nucleoprotein solution (4.0 ml). After a further 60 minutes, 4 ml of the mixture were placed in the viscometer, the temperature brought to 34.5° and the deoxyribonuclease added.

(c) An ethanolic solution of the nitrogen mustard (4.0 mg) was diluted with buffer (4.0 ml) and the nucleoprotein solution (8.0 ml) added with mechanical stirring. After 2 hours at room temperature, ethanol (3 vols) was added and the resulting precipitate collected at the centrifuge. A solution of the precipitate in distilled water (16.0 ml) and 0.1 *M* veronal buffer pH 7.1 (10.0 ml) was brought to 37° and deoxyribonuclease (0.02 per cent, ($^{w}/_{v}$) 4 ml) added. At suitable intervals, aliquots (3 ml) of the solution were withdrawn, cooled to 0° and 5 N hydrochloric acid (0.1 ml) added. The resulting precipitate was removed (centrifuge) and the total "acid soluble"

organic phosphorus determined in the clear supernatant as previously described.

The results of these experiments show that no inhibition of the enzymic degradation of the deoxyribonucleohistone resulted from the presence of colchicine, aminopterin or the -SH reactants (Fig. 7). On the other hand, the nucleoprotein, by reaction with the nitrogen mustards, became less susceptible to hydrolysis with deoxyribonuclease, although, under the conditions of the experiments, neither the depolymerisation (Fig. 8) nor the hydrolysis (Fig. 9) of the substrate were completely inhibited. Furthermore, the inhibitory activities of these substances were reduced considerably when they were kept in aqueous solution for 20 minutes before reaction with the nucleoprotein (Fig. 8).

DISCUSSION

The fact that the magnesium ion is essential for the activity of deoxyribonuclease from pancreas, has been observed by Fischer, Lehmann-Echternacht and Bottger (15), McCarty (28), Greenstein, Carter and Chalkley (19) and by Overend and Webb (31), (but cf. B. 2.). It follows therefore, that substances which inhibit the activity of deoxyribonuclease may be divided into two groups, namely those which exert their action through their ability to remove, by coordination or precipitation, the activating magnesium ion, and those which directly interact with the functional groups of the enzyme protein.

Among substances of the first group, we have confirmed in agreement with McCarty (28) and others, the inhibitory action of fluorides, citrates and borates. Reference to Fig. 1 shows that a final concentration of 1.67×10^{-2} *M* potassium fluoride or sodium borate completely inhibits the action of the enzyme on deoxyribonucleic acid in the presence of 1.67×10^{-2} *M* magnesium sulphate. Sodium citrate at the same molar concentration is only slightly less effective and gives 90 per cent inhibition during the same interval of time. Lower concentrations of potassium fluoride or sodium citrate (Fig. 1), which only partially remove the magnesium ion, decrease the activity of the enzyme without causing complete inhibition. The inhibitory action of arsenate and selenite (Table I) on the activity of the enzyme, may also reside in the ability of these ions to form sparingly soluble magnesium salts. Addition of a solution of sodium selenite to magnesium sulphate, for example, results in rapid crystallisation of magnesium selenite from solution.

Among substances of the second group, sodium sulphide and thioglycollic acid (Table I) were found to decrease markedly the activity of deoxyribonuclease. On the other hand, cysteine, sodium bisulphite, hydroxylamine and semicarbazide had no influence upon the activity of the enzyme. Similar negative results were obtained with the sulphydryl reactants, iodoacetamide and iodoacetic acid. These substances have been shown to arrest mitosis in telophase in cells in chick tissue cultures (23) and were, in consequence, of particular interest in this investigation.

The nature of the marked inhibitory action of copper and zinc ions upon the activity of the enzyme remains to be determined. In view of the lack of effect of iodoacetamide and iodoacetic acid (Fig. 1 and Table I) it does not appear that the action of these metals can be due to their reaction with essential -SH groupings of the enzyme. It is possible that these ions exert their effect on the nucleic acid rather than on the enzyme, since these and other metallic ions are known to precipitate deoxyribonucleic acid (15). The observation that zinc inhibits the enzymic degradation of deoxyribonucleic acid is of particular interest when considered in relation to the findings that significant quantities of zinc are concentrated in the deoxyribonucleoproteins isolated from thymus and tumour tissue (20). It is also interesting to recall that both copper and zinc inhibit the action of ribonuclease on ribonucleic acid, although this enzyme is inactivated by -SH reactants and presumably contains essential, functional sulphydryl groups (38).

The action of the enzyme on deoxyribonucleohistone proceeds in the absence of free magnesium ion. The inhibition of the enzyme by fluoride citrate, arsenate and selenite ions (Figs. 5 and 6) under these conditions, however, suggests that the enzyme-substrate system is activated by the magnesium present in the nucleoprotein. This observation is in accordance with the suggestion of Weissman and Fisher (36) that magnesium does not directly activate deoxyribonuclease, but alters the substrate in some way such that the enzyme may function.

Cohen (7) has shown that when deoxyribonucleohistone is digested with trypsin, there is an increase in the viscosity of the solution due to the liberated deoxyribonucleic acid exhibiting a higher viscosity than the nucleoprotein. In the present experiments, the rapid decrease in viscosity which occurred when deoxyribonuclease was added to the deoxyribonucleohistone solution commenced instantaneously, and an initial rise in viscosity was never observed. Thus, it has not been possible to determine whether the enzyme acts directly upon the nucleoprotein, or whether the degradation

of the deoxyribonucleic acid moiety is preceded by the dissociation of the nucleohistone.

The action of deoxyribonuclease on deoxyribonucleohistone was not inhibited by pre-treatment of the substrate with colchicine, 4-aminopteroylglutamic acid, or the -SH reactants chloroacetophenone, iodoacetic acid and iodoacetamide. The absence of any inhibitory action of the latter group of substances is in accordance with the apparent low sulphydryl content of the nucleohistone, and suggests that, under the given experimental conditions, these agents do not react with essential amino groupings (3).

The nitrogen mustards, which in sub-lethal concentrations cause mitotic abnormalities similar to those produced by mustard gas (17, 5, 12, 13), were observed to react with the nucleohistone and render the latter partially resistant to enzymic degradation. These compounds were added to the substrate in concentrated ethanolic solution; a method similar to that used by Dixon and Needham (10), in their studies on the action of mustard gas on various enzyme systems (21). Under these conditions some precipitation of the nucleoprotein occurred. Precipitation also results from the application of mustard gas to nucleoproteins (4) and nucleic acids (11). In the latter case, it is known from electrometric titration that reaction occurs between the mustard gas and primary and secondary phosphoryl groups as well as amino groups of the nucleic acid (11). It is probable that the effect of the nitrogen mustards is due to the alkylating effect of the halogenoalkyl groups on similar groups of the nucleic acid or nucleoprotein (18, 27, 6).

In view of the high order of reactivity of the nitrogen mustards with various functional constituents of the cell, little importance can be attached to the observations that the products from the interaction of deoxyribonucleohistone with these substances are resistant to enzymic attack. Indeed, with regard to the inhibition of mitosis produced by these compounds, their precipitating action on the nucleoprotein may be of greater significance than the fact that they render the latter partially resistant to degradation with deoxyribonuclease.

Apart from fluoride and selenite ions, which inhibit both deoxyribonuclease and mitosis in tissue cultures (23), the lack of effect of the various mitotic inhibitors on the degradation of deoxyribonucleohistone with deoxyribonuclease suggests that if an enzyme of the deoxyribonuclease-type exists in cell nuclei and plays any part in the sequence of changes which occur during mitosis, the properties of such an enzyme must differ considerably from those of the exocellular, pancreatic deoxyribonuclease.

SUMMARY

The action of inhibitors on the enzymic degradation of deoxyribonucleic acid and deoxyribonucleohistone by pancreatic deoxyribonuclease has been studied. The results show that the inhibitors may be divided broadly into two classes, namely those which remove the activating magnesium ion, and those which directly interact with the enzyme protein. Active inhibitors prevent both stages (i.e. depolymerisation and hydrolysis) of the degradation of the deoxyribonucleic acid.

The products which result from the reaction of nitrogen mustards with deoxyribonucleohistone are partially resistant to the action of deoxyribonuclease. Pre-treatment of deoxyribonucleohistone with other mitotic inhibitors has no effect upon its subsequent degradation with deoxyribonuclease.

Thanks are due to Professor M. Stacey, F.R.S. and to Dr. Honor B. Fell for their interest in this work, and to Dr. A. F. W. Hughes for suggesting the study of the action of mitotic inhibitors on the enzymic degradation of deoxyribonucleohistone.

REFERENCES

1. Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940).
2. Bargoni, N., *Boll. soc. ital. sper.*, **20**, 534, 1945. *Chem. Abs.* **40**, 6513 (1946).
3. Barron, E. G. S., and Singer, T. P., *J. biol. Chem.* **157**, 221 (1945).
4. Berenblum, I., and Schoental, R., *Nature*, **159**, 727 (1947).
5. Bodenstein, D., *J. Exptl. Zool.*, **104**, 311 (1947).
6. Butler, J. A. V., *Nature*, **166**, 18 (1950).
7. Cohen, S. S., *J. Biol. Chem.*, **158**, 255 (1945).
8. Davidson, J. N., *Ann. Rev. Biochem.*, **18**, 155 (1949).
9. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
10. Dixon, M., and Needham, D. M., *Nature*, **158**, 432 (1946).
11. Elmore, D. E., Gulland, J. M., Jordan, D. O., and Taylor, H. F. W., *Biochem. J.* **42**, 308 (1948).
12. Fell, H. B., and Allsopp, C. B., *Cancer Res.*, **8**, 145 (1948).
13. —— *Cancer Res.*, **8**, 177 (1948).
14. Fischer, F. G., Bottger, I., and Lehmann-Echternacht, H., *Z. physiol. Chem.*, **271**, 246 (1941).
15. Fischer, F. G., Lehmann-Echternacht, H., and Bottger, I., *J. prakt. Chem.*, **158**, 79 (1941).
16. Gilbert, L. M., Overend, W. G., and Webb, M., *Exptl. Cell. Res*, **2**, 138 (1951).
17. Gillette, R., and Bodenstein, D., *J. Exptl. Zool.*, **103**, 1 (1946).
18. Goldacre, R. J., Loveless, A., and Ross, W. C. J., *Nature*, **163**, 667 (1949).
19. Greenstein, J. P., Carter, C. E., and Chalkley, H. W., *Cold Spring Harbor Symp. Quant. Biol.*, **12**, 64 (1947).
20. Heath, J. C., *Nature*, **164**, 1055 (1949).
21. Herriot, R. M., Anson, M. L., and Northrop, J. H., *J. Gen. Physiol.*, **30**, 185 (1946).
22. Holmes, B. E., *Ann. Rep. Brit. Empire Cancer Campaign*, **26**, 191 (1948).
23. Hughes, A. F. W., *J. Roy. Microscop. Soc.*, **69**, 215 (1949); *Quant. J. Micro. Soc.* (in the press).

24. Jacobson, W., and Webb, M., *J. Physiol.*, **112**, 29 (1951).
25. Jungner, G., Jungner, I., and Allgen, L. G., *Nature*, **164**, 1009 (1949).
26. Koch, J. R., Orthmann, A. C., and Degenfelder, M. A., *J. Amer. Leather. Chem. Assoc.*, **34**, 489 (1939).
27. Kon, G. A. R., and Roberts, J. J., *J. Chem. Soc.*, **978** (1950).
28. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
29. Mirsky, A. E., and Pollister, A. W., *Proc. Natl. Acad. Sci.*, **28**, 344 (1942).
30. Overend, W. G., and Webb, M., *Research*, **2**, 99 (1949).
31. —— *J. Chem. Soc.*, **2746** (1950).
32. —— *Research*, **3**, 238 (1950).
33. Stern, K. G., *Exptl. Cell Res. Suppl. I*, 97 (1949).
34. Vercauteren, R., *Nature*, **165**, 603 (1950).
35. Webb, M., *J. Gen. Microbiol.*, **2**, 275 (1948).
36. Weissman, N., and Fischer, J., *J. biol. Chem.*, **178**, 1007 (1949).
37. Zamenhof, S., and Chargaff, E., *J. biol. Chem.*, **180**, 727 (1949).
38. Zittle, C. A., *J. biol. Chem.*, **163**, 111 (1946).

THE CHANGING CELL NUMBER AND COMPOSITION OF CHICK HEART EXPLANTS GROWING IN VITRO

J. N. DAVIDSON and I. LESLIE[1]

Biochemistry Department, The University, Glasgow, Scotland

Received October 10, 1950

The previous work by the authors on the chemical composition of chick heart fibroblasts growing in roller tubes has been confined to a study of the changes in the ribonucleic acid phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP) content of the cultures (6). Although an increase in the RNAP content of the 24 explants in each roller tube could be recorded under a variety of conditions, significant increases in the DNAP content could only be produced when the initial size of the explants was sufficiently small to allow the outgrowth of cells to exceed losses caused by degeneration in the central areas of the explants. Consequently by using relatively small explants it was possible to show increases as large as 160 per cent in the DNAP content of the cultures with corresponding increases in the RNAP content of about 350 per cent.

A characteristic feature of growth under these conditions was that the RNAP content would increase to a considerable extent before there was any rise in the DNAP. This occurred within the first eight hours in short term experiments, and in longer tests the RNAP would continue to increase for as much as 3 days before any rise in DNAP could be observed. Confirmation of these changes in the RNAP and DNAP of fibroblast cultures has recently come from the work of Signorotti, Hull and Kirk (17) and Hull and Kirk (10, 11, 12). However, these authors suggest that the preliminary rise in RNAP may not be a major factor in normal growth, but rather represents a replacement of RNAP lost during the starvation of the tissue in Tyrode solution. While our results are in agreement with this view that RNAP is lost from the cells when they are kept in Tyrode solution or Tyrode-serum, they also show clearly that the RNAP content of rapidly growing cells is higher than that found in cells of fully grown cultures or in cells of the original embryo heart tissue.

Two developments have made it possible to investigate more thoroughly the chemical changes which occur during the growth of these explants. The first is an improvement in the technique involving an increase in the number of explants per roller tube from 24 to 48, while at the same time largely eliminating the plasma clot in which cultures are generally grown. This is achieved by using a film of plasma in the roller tubes just sufficient to fix the explants to the wall. As the amounts of both phosphorus and nitrogen constituents in this film are extremely small or negligible in comparison to the material in the 48 explants, we are able to compare changes in the protein content of the explants with changes in the amounts of various phosphorus constituents.

[1] Imperial Chemical Industries Research Fellow.

With a large number of explants growing in each roller tube, the increase in their area ensures that the whole wall is covered with living cells in a matter of four or five days. Under such conditions it was thought that it might be possible to avoid any significant contamination of the culture tubes by protein and other materials present in the embryo extract used as the growth-promoting medium. However, plasma tubes containing no explants have been used in all the experiments described in this paper to enable us to compare the actual changes recorded for the culture tubes with those obtained after correction has been made for the plasma blanks. As a means of reducing the contamination to a minimum, both the explant and the plasma tubes were incubated at 37° C in Tyrode-serum for 24 hours after the growth period. This removed some of the deposited material from the plasma tubes; it also ensured that the explants were taken for chemical analysis from the same medium (Tyrode-serum) as the original explants at the start of the experiment.

The second development is concerned with the procedure of analysing and interpreting the chemical changes recorded during tissue growth. By the discovery and confirmation that the deoxyribonucleic acid (DNA) content of the nucleus is constant for the cells of the tissues of a given species (19, 20, 13, 5) it has become possible on the basis of chemical determinations to follow changes in cell number and cell composition in growing tissues. We have already shown (3, 4) how this procedure could be applied to both tissue culture and embryonic studies. In this present work the revised DNAP content of the nucleus of the somatic tissues of the fowl has been taken as 2.35×10^{-7} μg, and used as a basis for calculating cell number and for showing how the actual amounts per cell of other constituents vary throughout growth.

Since it is now generally accepted that DNA is an integral part of the chromosome structure, it must, of course, increase to twice its normal amount per cell at some stage prior to cell division. This may have little effect on cell composition as obtained by reference to a unit of DNA, for it is likely that there are corresponding increases in other constituents in the process leading to cell division. In rapidly growing tissues, however, it will mean that estimates of cell number based on DNA content will be slightly higher than the actual number of cells present in the tissue. Where we have introduced the practice of keeping the cultures in Tyrode-serum for 24 hours after the growth period, the errors in our estimates from this source should be reduced to a minimum.

METHODS

Since a detailed description of the general tissue culture technique has already been published (6), the following account will be concerned mainly with important modifications which have been introduced in the course of the present investigations.

Roller tubes. Each experiment involved the use of 20 roller tubes each containing 48 fresh explants and another 20 tubes containing only the plasma film clotted by a drop of sterile thrombin solution. The plasma film was formed by spreading about 0.05 ml of fowl plasma over the wall of the roller tube with the aid of a platinum wire. The stoppered tubes were then stood vertically for at least four hours to allow the excess plasma to drain to the bottom for removal with a capillary pipette. The tubes were stoppered and kept in the cold until required (usually overnight).

Explants. Fresh explants were obtained as before from 12 day chick-embryo

hearts. Throughout the whole process of cutting the hearts into the small pieces required for planting, the tissue was immersed in a Tyrode-serum mixture (2:1). Forty-eight pieces were planted rapidly in 8 rows of 6 each, and the rubber stopper replaced immediately to prevent loss of moisture. Even so the tissue explants deteriorated unless the tubes were rotated with 1 ml Tyrode-serum at 37–38° within 15 to 25 minutes of planting. This entailed leaving the explants just long enough in contact with the plasma film to allow them to adhere to the wall before adding the Tyrode-serum. At this stage each roller tube of 48 explants contained about 15 mg fresh weight of heart tissue. These explants normally remained for 24 hours in the Tyrode-serum at 37° before growth-promoting medium was substituted as fluid phase.

Growth-promoting medium. In past work we followed the usual practice of preparing the embryo extract under ice-cold conditions. We have since obtained much better results in terms of more vigorous growth and reduced contamination by using an embryo extract prepared by incubating the crushed chick embryos in Tyrode solution at 37° C for 24 hours. (This procedure was adopted as a result of the demonstration by Sanford, Earle and Likely (15) of the beneficial effect on culture growth from single cells obtained by the use of embryo extract, previously 'conditioned' by contact with growing cultures for 24 hours.) After centrifuging the pulped embryos for 20 minutes at 3 000 r.p.m., the supernatant fluid was collected and yielded about 7 ml of concentrated extract from each embryo. This was usually diluted with Tyrode solution to 10 ml, and the final test extract produced by adding a further 5 ml of Tyrode-serum (2:1), giving a total N concentration of between 70 and 100 mg per 200 ml. The incubated extract alone without added serum seemed to serve equally well as a growth-promoting medium, but it was our practice to use the incubated extract-serum (IES) to give the cultures every chance of remaining in a healthy condition throughout growth.

Test procedure. The material present in the 48 explants after they have been incubated for 24 hours at 37° C serves as a base line for measuring the subsequent increases during growth. Throughout the tests the 1 ml of growth-promoting agent (IES) in each roller tube was replaced by a fresh lot every 24 hours. Growth, as determined by DNAP increases, was somewhat less extensive if the IES was renewed at 48 hourly intervals. The tubes were prepared for chemical analysis by extracting the fluid and rapidly washing out each tube with non-sterile physiological saline. In the case of explants sampled at the time of planting or in the early stages of growth, it was necessary to combine the contents of two or more roller tubes to ensure that there was at least 1.5 μg DNAP in the N NaOH used in separating RNAP from DNAP by the Schmidt and Thannhauser (16) method. With less DNA there was a danger of incomplete separation. At later stages it was possible to carry out the complete chemical analysis on the contents of a single roller tube, and to base results on separate duplicate determinations. The corresponding plasma blank tubes were treated in exactly the same way as the culture tubes.

Chemical analyses. The procedure of fractionating the various phosphorus constituents of the tissue followed closely the one described in our previous paper (6). The only modification concerns the determination of protein N in the residue left after extraction of acid-soluble (10 per cent trichloracetic acid) material and lipids. This residue was incubated as before in N NaOH overnight at 37°, but one half was

taken for N determination by the micro-Kjeldahl technique. It was found most convenient and reliable to oxidise the protein with 0.5 ml 60 per cent perchloric acid by heating in an air-bath at 210° C until the solution was clear and colourless. The whole process of evaporation (on air-bath at 120°) and oxidation (air-bath at 210°) was carried out in wide Pyrex boiling-tubes, which were covered with glass-bulb condensers to retain the perchloric acid fumes. Ogur and Rosen (14) have reported that they found oxidation with perchloric acid to be unreliable in N determinations, owing possibly to losses through decomposition of ammonium perchlorate. In our experience low returns will be produced by over-heating of the solution during oxidation or by an occasional faulty sample of perchloric acid, but with the procedure which we have described, recoveries can be obtained accurate to within ± 2 per cent.

The phosphorus content of the acid-soluble material, of the lipid fraction, and of RNA and DNA was determined by the Berenblum and Chain (1) method as modified by Davidson and Waymouth (7). All results are expressed as μg P or N per 48 explants or per roller tube in the case of the plasma blanks. The figures for protein N are obtained by subtracting from the residual N the corresponding amounts of nucleic acid N, estimated by multiplying the total nucleic acid P by 1.69. Those estimates are close approximations based on the relative amounts of P and N in yeast ribonucleic acid and in thymus deoxyribonucleic acid.

RESULTS

Our earlier work (6) showed that the RNAP and DNAP contents of chick heart explants fell appreciably during the 24 hours following planting. If the fluid medium consisted of Tyrode solution (which became mixed with serum from the relatively large plasma clots) the percentage decrease in both components was rather larger than was the case if embryo extract was added immediately after planting. These losses, we suggested, were the result of the extensive breakdown of tissue damaged during the cutting process.

With the new technique we have been able to follow the changes in amounts of protein nitrogen (PN), acid-soluble phosphorus (ASP), and lipid phosphorus (LP), as well as RNAP and DNAP. Fig. 1 shows what happened in a series of tissue culture tests when the explants were incubated with Tyrode-serum for 24 hours after planting. The left-hand section of the diagram gives the actual amounts of the various constituents present in 48 explants at the time of planting (PT) and after 24 hours in Tyrode-serum. These results have been corrected for plasma blanks, which are shown as dotted lines below; no DNAP was found in the plasma blanks in any of the tests. Under these conditions the main constituents of the explants, with the exception of the ASP, are greatly reduced in amount. The ASP and PN are distinguished by the relatively high amounts found in the

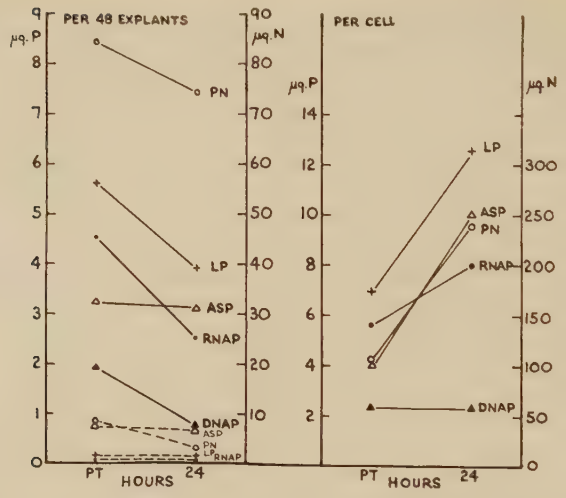


Fig. 1. Chemical changes in chick heart explants during 24 hours in TS immediately following planting in roller tubes. Left section—amounts of constituents (after correction) per 48 explants or per roller tube in case of plasma blanks. Results for 48 explants—unbroken lines; plasma blank results—broken lines. Right section shows content of various constituents per cell based on DNAP content per cell of 2.35×10^{-7} μg. PT-planting time. All other abbreviations as shown in Table I.

plasma blanks as compared with the negligible amounts of the other constituents. The ASP in the blank amounts to 25 per cent of that present in the explants, and the protein remains less than 10 per cent of the explant protein.

From the decrease in the amount of DNAP per 48 explants we can deduce that the number of cells has diminished to about one-third of their original number. When we express the results as amounts per cell (taking the DNAP content of the chick cell nucleus as 2.35×10^{-7} μg), we see the relative changes in tissue composition which accompany these losses in material. These are illustrated on the right hand section of Fig. 1. Three of the constituents, ASP, LP and PN increase their content per unit of DNAP by 100 per cent or more; the RNAP in contrast rises only 30 per cent. Examination under the microscope at this stage (i.e. after 24 hours in Tyrode-serum) usually revealed a narrow band of migrating cells round the explants.

In one set of explant tubes we compared the effects of adding TS and IES to the explants immediately after planting (Fig. 2). The left-hand section of the diagram shows that the losses in material were much greater in the case of Tyrode-serum (TS), and that the IES by reason of its growth-promoting activity was able to diminish, but not prevent, these losses. When

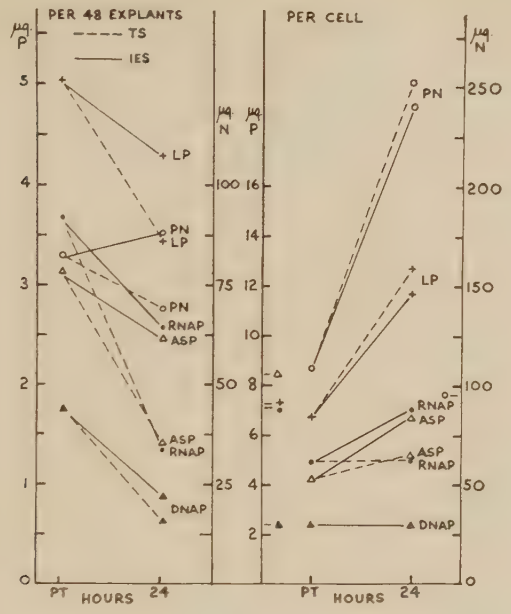


Fig. 2. Comparison of chemical changes in explants when in contact with either TS or IES during the 24 hours immediately following planting in roller tubes. Left section—amounts of constituents per 48 explants after correction for plasma blanks. Right section—content per cell for the same constituents (as in Fig. 1). Unbroken lines—explants in contact with IES; broken lines—explants in TS. PT—planting time. All other abbreviations as in Table 1.

the changing composition of these explants is examined (right-hand section), it is clear that the type of fluid medium has had little effect on the amounts of LP and PN per unit of DNAP, for at the end of 24 hours they are increased to the same extent by both treatments. The presence of growth-promoting medium has, however, increased the amounts of RNAP and ASP per cell, which in this test were little changed during the 24 hours in Tyrode-serum.

From these results it is evident that a striking change in explant composition occurs in the 24 hours following planting. In relation to the constant DNAP, the PN and LP contents are more than doubled whatever the medium. Because of the relative quantities involved this cannot be the result of retention of material from the plasma or of contamination from the fluid medium; it is concluded that these increases are part of the activity of the explants and that the additional material in the cells comes from damaged cells or from the fluid phase. The RNAP and ASP also appear to be closely associated fractions. In TS they will increase per cell or remain unchanged

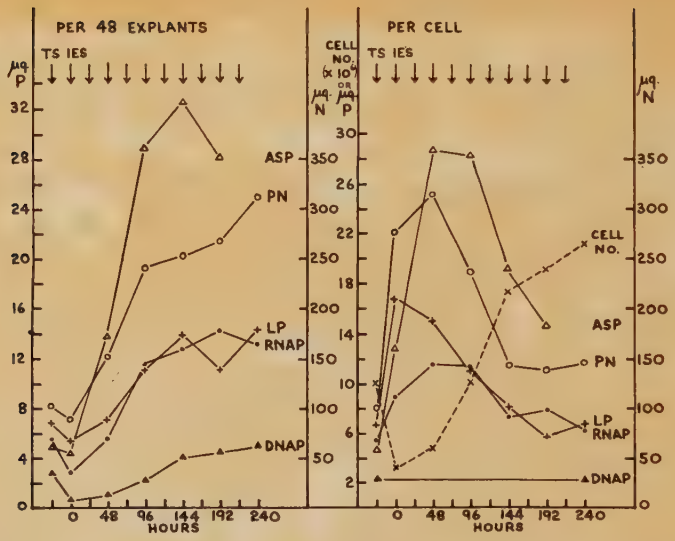


Fig. 3. Chemical changes in chick heart explants grown *in vitro* during Test G 17. Left section —amounts per roller tube of 48 explants without correction for plasma blanks. Right section—amounts per cell without correction for plasma blanks (based on DNAP as for Fig. 1). Unbroken lines show amounts of constituents; broken line shows cell number. Hours indicate time during which explants were in contact with TS or IES; they were treated with TS for 24 hours after each period of growth in IES. All abbreviations as for Table I.

throughout 24 hours, while the addition of growth-promoting medium (IES) increases their amounts per cell over those found in the case of TS.

Cell number and cell composition during growth. In all our experiments on the effect of growth-promoting agent on cell number and cell composition of these explants, it has been our practice to keep the cultures in TS for the first 24 hours in order to establish a base-line for subsequent measurements. Fig. 3 contains the results of a single test, which is typical of many that have been carried out. In the left hand section the dotted lines between the points at planting time (PT) and zero time (ZT) show how the amounts of the various fractions per 48 cultures have altered in Tyrode-serum, and how their absolute values are very much smaller than those obtained at later stages of the test. In this graph no correction has been made for plasma blanks, and the results represent the actual amounts present per roller tube of 48 explants. Every 24 hours throughout growth (as indicated by the arrows) the IES was replaced by fresh 1 ml lots. When tubes were taken for chemical analysis in this series of experiments, they were incubated after their growth period for an additional 24 hours in con-

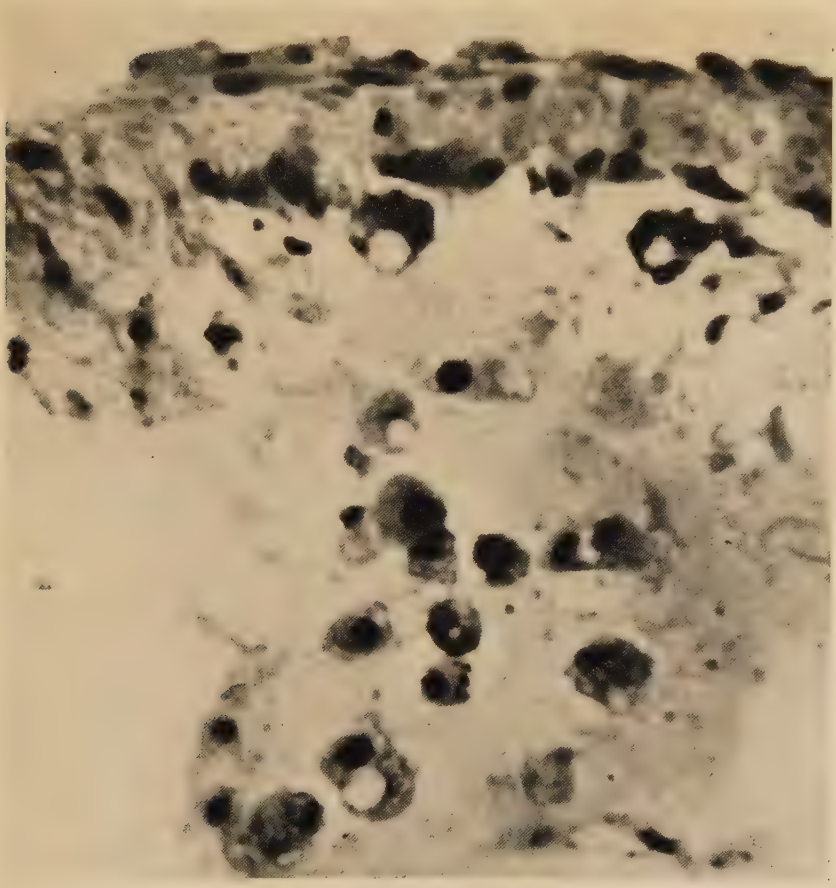


Plate I.

Transformation of fibroblasts into macrophages in 10 day cultures of chick heart explants growing in roller tubes. Observation of cultures in the roller tube under low-power microscope showed appearance of dark round cells in areas where growing explants had overlapped. Paraffin sections taken from sheets of cells embedded in plasma clot, and fixed in formol-saline; this section stained with ironhaematoxylin and eosin. Magnification × 800.

tact with 1 ml of Tyrode-serum. Preliminary experiments had shown that this produced only small changes in the number or composition of the culture cells, and that it helped to reduce the plasma blanks.

The increase in DNAP is most pronounced between 48 and 144 hours of growth, and is succeeded by a slower rise during the last four days of the test. By 144 hours the culture cells had covered the entire surface of the roller tube, and further expansion could only arise by increases in the

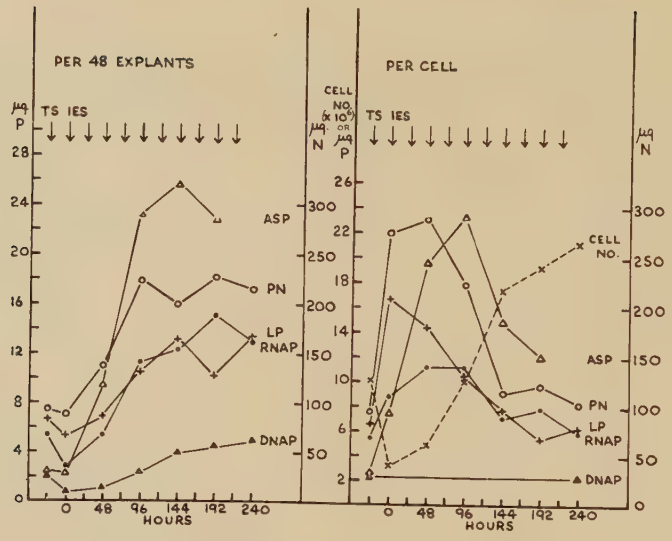


Fig. 4. Chemical changes in chick heart explants grown *in vitro* during Test G 17 after amounts per 48 explants have been corrected for corresponding plasma blanks. Otherwise, as for Fig. 3.

number of layers or in the density of the cells. From microscopic examination it appeared that many layers of cells had formed, and that cells quite different in appearance from the usual spindle-shaped fibroblasts became increasingly prominent during the last four days of growth. A photomicrograph of a section of these cultures (Plate I) shows the distinction between the two types; one, spindle-shaped, is the normal fibroblast, the others, irregular in shape and sometimes vacuolated, have the characteristics of macrophages. None of these was seen in plasma blank tubes (as confirmed by absence of any significant increase in DNAP in Table I), and they could not have come from the embryo extract. These results confirm the earlier observations of Ephrussi and Hugues (8) and others that the transformation of fibroblasts to macrophages will occur during growth in vitro.

While the DNAP (and, therefore, cell number) continued to increase rapidly until 144 hours, the greatest accumulation of PN, ASP, RNAP and LP took place in the first 96 hours. Any further increases in these constituents were small by comparison, and in the case of PN may have been caused by contamination from the IES, which produced in the plasma blanks PN values at 144 to 240 hours representing between 14 and 30 per cent of the amounts found in the tissue culture tubes (Table I).

TABLE I

Results of analyses of contents of roller tubes in Test G 17. Figures for plasma blanks are actual amounts found per roller tube. Those for 48 explants have been corrected for amounts found in corresponding plasma blank tubes. In each case results are expressed as μg P or μg N. ASP—acid soluble phosphorus; LP—lipid phosphorus; RNAP—ribonucleic acid phosphorus; DNAP—deoxyribonucleic acid phosphorus; NAP—total nucleic acid phosphorus; PN—protein nitrogen; TS—2:1 mixture of Tyrode solution and fowl serum; IES—mixture of incubated embryo extract and fowl serum. The time is given in hours during which explants or plasma films were in contact with TS or IES.

Roller Tube	Fluid phase	Time hours	μg P or N per roller tube						
			ASP	LP	RNAP	DNAP	NAP	P	PN
Plasma blank	—	—	2.29	0.14	0.07	0.00	0.07	2.5	7
» »	TS	24	1.74	0.03	0.03	0.00	0.03	1.8	0
» »	IES	+ 48	4.31	0.05	0.16	0.00	0.16	4.5	13
» »	IES	+ 96	5.52	0.63	0.06	0.00	0.06	6.2	27
» »	IES	+144	9.72	0.67	0.46	0.00	0.46	10.9	52
» »	IES	+192	5.12	0.75	0.05	0.06	0.11	6.0	38
» »	IES	+240	—	0.94	0.16	0.00	0.16	—	98
48 Explants in plasma	—	—	2.50	6.66	5.44	2.38	7.82	16.98	94
48 Explants	TS	24	2.39	5.38	2.86	0.76	3.62	11.39	89
48 »	IES	+ 48	9.43	6.95	5.36	1.13	6.49	18.55	138
48 »	IES	+ 96	23.2	10.5	11.37	2.39	13.76	47.46	223
48 »	IES	+144	25.6	13.2	12.4	4.1	16.5	55.3	200
48 »	IES	+192	22.8	10.3	15.1	4.5	19.6	52.7	228
48 »	IES	+240	—	13.4	13.1	5.0	18.1	—	215

The results for the same test after correction has been made for the plasma blanks are given on the left of Fig. 4. The amounts of DNAP, RNAP and LP remain much the same as before correction, and the main alterations are confined to reductions in the amounts of PN and ASP. In these circumstances the PN of 48 cultures appears to have reached its maximum at 96 hours, and to have remained at this level in spite of the more than twofold increase in cell number which followed during the next six days.

Calculation of cell numbers on the basis of the constant DNA content of the nucleus shows that two-thirds of the cells present at the time of planting were lost in the first 24 hours in TS (Table II). Between 48 and 144 hours the numbers approximately doubled in each 48 hour period, followed by the slow rise to the maximum of 21 million per 48 explants. There were consequently seven times as many cells per roller tube at 240 hours as were present at zero time.

The changes in cell composition which accompany these successive phases

TABLE II

Cell number and protein nitrogen/phospholipid phosphorus (PN/LP) ratios during tissue culture growth. Cell number from test G 17 is calculated on the basis that the nuclei of all fowl somatic cells contain $2.35 \times 10^{-7} \times \mu g$. DNAP. PN/LP ratios refer to following tests—1. based on results of G 17 (Table I); 2. based on pooled results (Fig. 5). PN, LP, TS and IES as in Table I. The time is given in hours during which explants were in contact with TS or IES.

Explants	Medium	Time hours	Cell number ($\times 10^6$)	Ratios	
				PN/LP (1)	PN/LP (2)
48	—	—	10.1	13.7	14.1
48	TS	24	3.2	19.1	16.5
48	IES	48	4.8	18.1	19.9
48	IES	96	10.1	18.6	21.2
48	IES	144	17.3	18.5	15.1
48	IES	192	19.0	22.4	22.4
48	IES	240	21.2	16.1	16.1

of culture growth, are shown on the right of Figs. 3 and 4 which respectively give the results before and after correction has been made for plasma blanks. In each figure the cell number, expressed as millions per 48 cultures, is drawn as an unbroken line between the time of planting and zero time, and as a broken line for the rest of the test. The other lines show in contrast the changes in the amounts per cell of ASP, LP, RNAP and PN, when the DNAP is taken as constant at 2.35 μg per cell.

A comparison of the two figures (Figs. 3 and 4) makes it clear that the correction for the plasma blank makes no difference to the pattern of the changes in cell composition, although it does reduce the amounts of ASP and PN per cell considerably. In both cases the 24 hours in TS produced abrupt increases in PN and LP, which brought these constituents to near their maximal amounts per cell. After 48 hours both PN and LP were decreasing per cell until they reached a fairly constant level between 144 and 240 hours. On the other hand, ASP and RNAP showed more prolonged increases, only reaching their maximal amounts per cell between 48 and 96 hours. Later, the RNAP and, possibly, the ASP fell to a constant amount per cell in the last four days of growth.

The high values for ASP and RNAP per cell coincided with the phase of rapid multiplication of the cells. By the end of that phase the PN and LP of the cells had fallen to a final steady level, and the cells continued to multiply more slowly with little change in composition. Along the ordinates are points showing the amounts of the various constituents of the cells

TABLE III

Comparison of initial and final cell numbers per 48 chick heart explants in a series of tissue culture tests. Cell number is calculated on the basis that the nuclei of all fowl somatic cells contain 2.35×10^{-7} μg. DNAP. TS and IES as in Table I. The time is given in hours during which explants were in contact with TS or IES.

Ex-plants	Me-dium	Time hours	Cell Number ($\times 10^6$)					Ratios				
			1	2	3	4	5	1	2	3	4	5
48	TS	24	1.4	1.6	2.7	3.0	3.2	1	1	1	1	1
48	IES	144	7.6	11.5	13.6	14.5	17.3	5.4	7.2	5.1	4.6	5.4
48	IES	240	—	—	—	22.6[1]	21.2	—	—	—	7.5[1]	6.6

as determined in the original heart tissue from which the explants were prepared. Apart from the ASP fraction, there was little difference between the composition of the explants (after correction for plasma blanks) at 240 hours and the composition of the embryonic heart tissue.

In this culture technique one variable, which affects the extent of culture growth, is the initial size of the explants. In any one test the pieces will be of an average size which determines the amount of DNAP or number of cells per roller tube at zero time. It is, however, impossible to cut them to the same average size in every test, and the initial cell number varies from one test to another. Table III contains results for cell number at zero time and after 144 hours in contact with IES in five different tests. In all the cell number increased 6 or 7 times, the final numbers keeping in the same proportion to one another as those initially present. They illustrate that the growth of cultures under these conditions is proportional to the initial size of the explants, provided that they are small enough initially to prevent deterioration of the cells at the centre of the explants.

Although the amounts of the materials per 48 cultures may vary from one test to another, we have found that results expressed in terms of amounts per cell are closely similar for corresponding periods of growth. As a result it is possible to combine the results from a series of tests to produce a more generalised account of the changes in cell composition of chick heart explants. This is given in Fig. 5. It largely reproduces the pattern shown for one of the tests, but emphasises the close association between PN and LP on the one hand, and between ASP and RNAP on the other. The rise and fall in content of PN and LP per cell coincided, the ratio between the two having kept fairly constant throughout growth (Table II). As before, the

[1] At 192 hours.

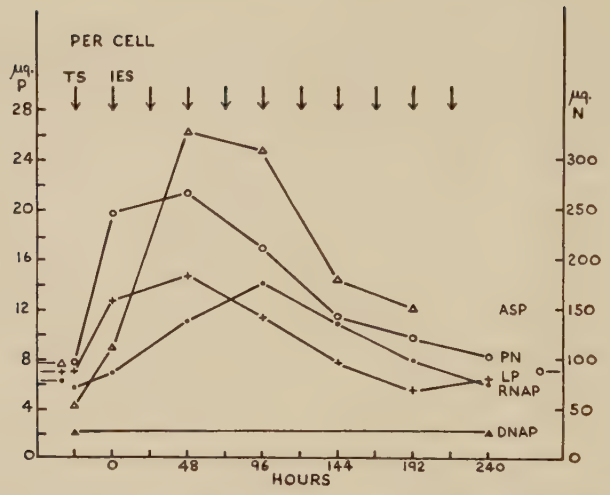


Fig. 5. Amounts per cell of constituents in 48 explants growing *in vitro*. Results are means obtained from a series of five tests, and all have been corrected for plasma blanks. Points along the ordinates show amounts per cell for the same constituents in the 12-day chick embryo heart from which the explants were obtained. Hours indicate time during which explants were in contact with TS or IES; they were treated with TS for 24 hours following each period of growth in IES. All abbreviations as for Table I.

ASP and RNAP attained their maximal amounts per cell over the period 48 to 96 hours.

Composition of explants in contact with growth-promoting medium. In the tests just described the explants have been kept for 24 hours in Tyrode-serum after their periods in contact with growth-promoting medium. This, we had found in preliminary experiments, greatly retarded the rate of growth, and produced only slight changes in composition. It gave us, therefore, information on the general composition of the cells in explants which were brought to a 'resting state' at each stage of their growth. How little this operation influenced the change in cell number and composition of the actively growing explants can be seen in the results of an experiment in which the explants were still in contact with IES at the time of sampling. As before the amounts of material in 48 explants in contact with TS for 24 hours were taken as the basis for measuring subsequent increases; the results shown in Fig. 6 have been corrected for plasma blanks, which were kept at a minimum by repeated centrifugation of the IES at 3 000 r.p.m. throughout the course of the test. On the left of Fig. 6 the final seven-fold increase in DNAP or cell number is the result of growth of the explants over the whole surface of the roller tube. Again the phase of rapid cell multiplica-

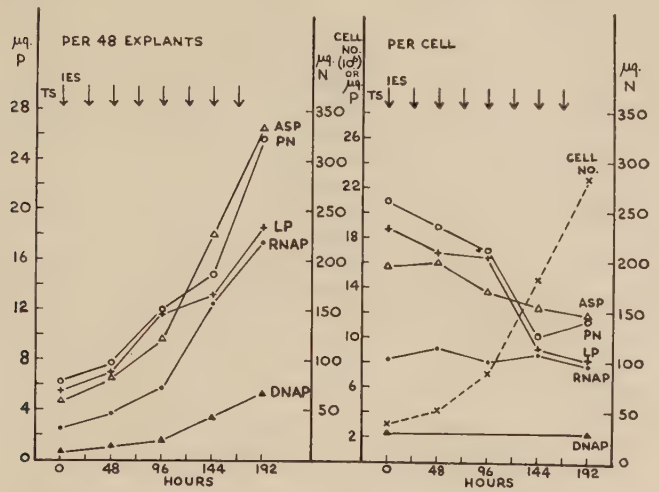


Fig. 6. Chemical changes in 48 chick heart explants grown *in vitro*, but in this test (G 18) explants were not kept in contact with TS for 24 hours following their growth period in IES, as in G 17 (Fig. 4). All results have been corrected for plasma blanks. Left section —amounts per 48 explants. Right section—amounts per cell based on DNAP content (as in Fig. 1). Unbroken lines—amounts of constituents; broken line—cell number. All abbreviations as for Table I.

tion, in this test from 96 to 144 hours, involved a two-fold increase in cell number over 48 hours.

Changes in cell composition can be seen on the right of Fig. 6, where they can be compared with the increases in cell number represented by the dotted line. These follow the same general pattern of a steep decline in the amounts of PN and LP over the phase of rapid growth, and a close association between the ASP and RNAP of the cells. In the normal chick heart from 12 day embryos we have found that the RNAP content per cell is on the average 6.25 μg. In this test it had already risen to 8.2 μg in the first 24 hours in TS, and it fluctuated in the region of this amount throughout the period in contact with growth-promoting medium. The highest value was 9.1 μg at 48 hours, and the lowest 7.7 μg at 192 hours. The trend is the same as before, but the IES has apparently prevented the decline in the RNAP per cell that was evident in explants brought to their 'resting state' in TS.

Effects of Tyrode-serum on growing explants. We have seen that there is little difference between the pattern of changes found in cultures sampled direct from IES and in those which remained in contact with TS for 24 hours after their growth phase. In both cases, high values for ASP and RNAP per cell were associated with rapid cell proliferation with the ten-

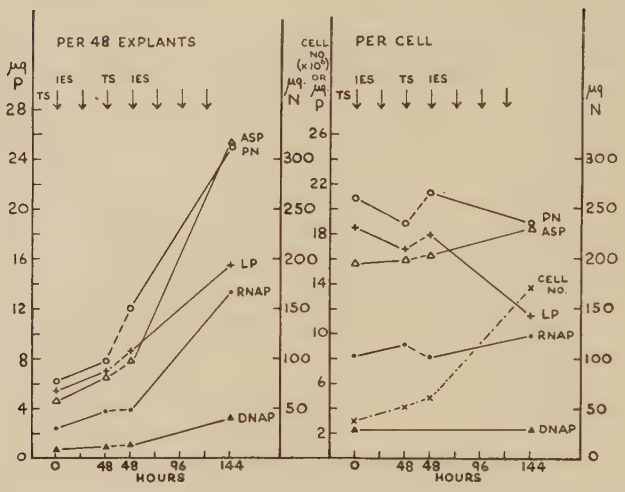


Fig. 7. Effect of keeping 48-hour growing chick heart explants in contact with TS at 37° for 24 hours before resuming growth in IES. Left section—amounts per 48 explants corrected for plasma blanks. Right section—corrected amounts per cell. Unbroken lines show amounts of constituents after periods of growth in IES; broken lines show amounts after 24 hours in TS. Dotted line gives changes in cell number. Scale of hours indicates time in contact with IES. All abbreviations as for Table I.

dency for these to decrease when growth-promoting medium was replaced by TS. We thought it desirable to investigate further the effect of Tyrode-serum on the cultures, and in particular, to find whether their ability to grow would be impaired by keeping partly grown cultures in TS for 24 to 48 hours before allowing them to grow again in IES.

The changes per roller tube produced by keeping 48 hour explants in TS for a further 24 hours can be seen in the left of Fig. 7. The amounts of the various constituents increased slightly, PN showing a rather larger increase than the rest. When cultures treated in this way were given a further spell of 96 hours in contact with IES, their final values for all the constituents left no doubt that normal growth had continued in the second phase. For this tube and its control (Fig. 6), which had spent an uninterrupted 144 hours in IES, the cell numbers were 13.6 and 14.5 millions respectively. The final RNAP and LP values were closely similar, but the ASP and PN were higher in the former than in the control. The right-hand section of Fig. 7 shows that the cell composition altered only slightly, if at all, during the 24 hour interval in TS. The fall in the RNAP per cell is perhaps significant by contrast with the increases in other constituents. As this slight change in composition occurred, the cell number rose from 4.1 to 4.8 million.

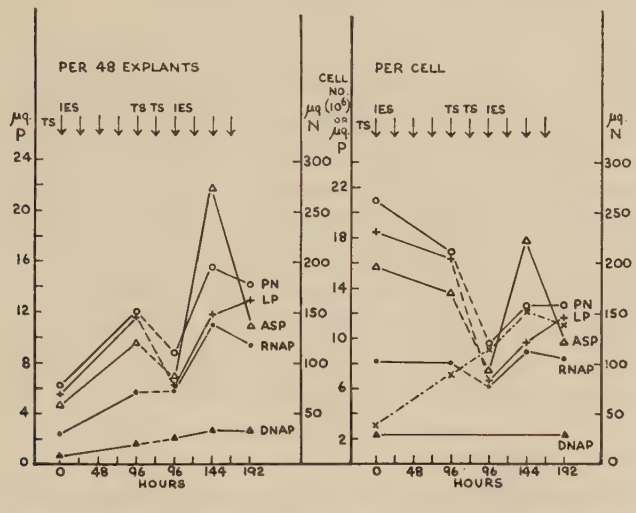


Fig. 8. Effect of keeping 96-hour chick heart explants in contact with TS at 37° for 48 hours before resuming growth in IES. Otherwise as for Fig. 7.

The effect of keeping 96 hour explants in TS for a 48 hour interval was investigated in a similar experiment. On the left of Fig. 8 are shown the amounts per 48 explants at different stages of the test. During the two days in TS, only the DNAP and RNAP increased or remained unchanged; in contrast the amounts of PN, LP and ASP decreased significantly. Although the cell number shown on the right of Fig. 8 increased from 7.1 to 9.2 millions in this time, it would have normally reached about 14 million had the cultures remained in contact with IES. Restoration of IES to the cultures produced a subsequent rise in cell number, but the final figures in the region of 11 to 12 million are much less than the 14.5 and 22.6 million found at similar stages in cultures taken from the same batch (fig. 6).

From the changes in cell composition, given on the right of Fig. 8, we can see that the cells at the end of the 48 hour interval contained about half the PN, LP and ASP that was present in the explant cell after 96 hours growth. However, in the cases of PN and LP, the same change (a fall in the amount of each per cell) occurred when the explants continued to grow in the normal manner in IES from 96 to 144 hours, the main difference being confined to the higher ASP and RNAP values in the rapidly growing cells. The return of the explants to growth-promoting medium caused slight increases in the amounts of the constituents per cell and resulted in limited growth.

DISCUSSION

If we consider first the changes in cell number of 48 explants as determined from the total DNAP content of the tissue, we find the following characteristic features of growth *in vitro*. There is in the period immediately following the planting of the cultures an unavoidable loss of the main cell constituents, and from the decrease in the DNAP we can deduce that as many as two-thirds of the cells are lost when the cultures remain for 24 hours in Tyrode-serum. It is also clear that this loss is only slightly diminished by keeping the cultures in contact with growth-promoting medium from the time of planting. In both cases the losses are rather larger than those we recorded in our previous paper (6), and this may be explained by the much smaller size of the explants, and the greater damage incurred during the cutting process.

When growth begins in response to the IES the cells continue to multiply rapidly for 6 to 8 days after which there is only a gradual increase in number. The cell number does not always double in the first 48 hours, but, in the next two 48 hour periods, we have found that this rate of cell multiplication is frequently obtained in cultures of 12 day chick heart explants. Similar growth rates for chick heart fibroblasts *in vitro* are recorded in earlier studies summarised by Fischer (9). It is perhaps significant that the growth rate of these explants is the same as the growth rate of cells of the chick heart between the 12th and 14th days of incubation (3, 4). This rate of cell increase clearly depends on the provision of an adequate growth-promoting medium; it was never exceeded in the presence of IES containing 4.5 to 6 mg of protein, that is roughly 6 to 10 times the amount of protein present in 48 explants. On the other hand cell multiplication was greatly retarded when the explants were in contact with serum containing about 3 mg protein for periods of 24 and 48 hours. In one test (Fig. 8) instead of the normal increase in cell number of 100 per cent over 48 hours, only a 30 per cent increase was obtained in the presence of Tyrode-serum. This occurred after the cultures had been growing in IES for 96 hours, and had reached the stage when their growth rate was normally at its peak.

The few results which we have for explants of different sizes (i.e. within the limits required to obtain appreciable growth) show that the number of cells present when growth is complete or nearly so, is directly proportional to the number in 48 explants at the start of the growth period. We have never obtained an increase greater than six or sevenfold, but this limit was reached whether we started with about 1 million or with 3 million cells.

The time required for the cultures to grow to their maximum size varied between 6 and 10 days, but since these tests were carried out at different times with different batches of material it is not possible to decide how far the initial size affected the rate of growth. Hull and Kirk (10, 11, 12), have raised some doubts about earlier observations summarised by Fischer (9), which indicated that the larger the initial explant, the greater was the final area. This conclusion was also reached by Brues, Rathbun and Cohn (2) who noted much less migration and mitosis in small explants than in larger ones. Although in these earlier studies the relationship between cell number and the area of the explant has not been precisely defined, our results support the general concept that the number of cells initially in the explant determines the final number in fully grown explants.

It would be of some value at this stage to discuss how far changes in the chemical composition of cells growing in vitro will enable us to understand the chemical events which lie behind the multiplication of cells. It seems that an answer to this question should come from the study of the changing cell composition under conditions in which a large proportion of the cells are actively dividing without at the same time undergoing a permanent change in composition. This requires a system in which the cells in the fully grown explants are closely similar in composition to the original cells, since this will ensure that any changes in composition during growth will be part of the chemical process leading to cell division.

Such conditions are in fact found in our roller tube cultures of chick heart explants, in which the final cell composition is not significantly different from that of the cells in the original fragments of heart tissue (Table I). In the earlier stages of growth, too, the 70 000 to 150 000 cells in each of 48 explants double their number in a two-day interval. As the average time between one mitotic division and the next has been estimated as 10 hours (9), the proportion of cells approaching or undergoing mitosis must be appreciable in the rapid phase of growth. A detailed study of explants from 8 day chick heart by Tompkins, Cunningham and Kirk (18) showed 20 to 160 mitotic cells per 1 000 total cells in 24 and 48 hour cultures according to the region of the explant examined. In view of the relatively short duration of the actual mitosis, it is safe to assume that the proportion of cells whose composition is changing prior to division, will be appreciably larger than these figures for the proportions of mitotic cells might suggest. We consequently have good reason to conclude that the changes in composition of our cultures are related to the chemical processes leading to cell division.

The onset of growth was always accompanied by large increases in the amounts of protein and phospholipid per cell in the 24 hours following planting. The corresponding changes in the ASP and RNAP depended to a much greater extent on the nature of the medium; with Tyrode-serum their amounts per cell remained unaltered or increased slightly, but with IES the effect was to increase these amounts significantly. As the cultures entered their rapid growth phase after 48 hours, the protein and phospholipid content of the cells began to fall and reached their original levels at 144 hours or later. In contrast the ASP and RNAP per cell both remained at their high levels throughout the phase of rapid growth between 48 and 144 hours. All constituents returned to near their levels in the original cells of the heart tissue in the final slow phase of growth after 144 or 192 hours.

From this consistent pattern of changes we can draw two conclusions. First, there is in the phase before rapid cell division a two to threefold increase in the average cellular content of protein and phospholipid. This is strikingly supported by the determinations of average cell mass in similar cultures by Tompkins, Cunningham and Kirk (18). They found that the average cell mass in the peripheral growth areas of the explants was higher in 24 and 48 hour cultures than in cultures at 72 and 96 hours, and they concluded that these larger cells were two to four times the mass of cells in the centre of the explants. When it is remembered that the proportion of cells undergoing mitosis is also much greater in 24 and 48 hour cultures, there seems little doubt that the increased cell sizes are the result of increased protein and phospholipid per cell prior to division. For this reason, and because of the relatively slow synthesis of DNAP in the first 48 hours of growth, we conclude that the protein and phospholipid components of the cells are synthesized before the DNA of the chromosomes.

The second characteristic feature is the association between ASP and RNAP and the relatively high amounts of these constituents in the cells during the phase of rapid cell multiplication. The amounts of both per cell are still maximal at 96 hours, when there is already an appreciable decline in the protein and phospholipid content of the cells. This seems to suggest that ASP and RNAP have some part to play in the process of cell division which is distinct from the generally accepted association between RNA and protein synthesis; for, if we compare the percentage increases at 192 hours over the amount present per 48 explants at 96 hours in IES (Fig. 6), we find that the protein increased by 114 per cent, the RNAP by 207 per cent and the DNAP by 231 per cent. In other tests in which the cultures spent 24 hours after growth in Tyrode-serum, there was very little

protein (per 48 cultures) synthesised after 96 hours (Fig. 4), while the RNAP continued to increase as more DNA was synthesised. At the same time there was a reduction in the average RNAP content of the cells in this later phase which coincided with the lower rate of cell multiplication.

Further evidence which suggests that RNA is linked in some way to the synthesis of DNA comes from the experiments in which rapid growth was halted by keeping the cultures in Tyrode-serum for 24 and 48 hours. In both cases there was little or no increase in the total amounts of RNAP per 48 cultures, but there were 15 and 30 per cent increases in the total DNAP values. As a result we see (Fig. 7 and 8) that the synthesis of this DNA was accompanied by corresponding reductions in the average amounts of RNAP per cell. This, of course, does not necessarily involve a quantitative conversion of one to the other, but the fact that only the RNAP consistently behaved in this way suggests that the accumulation of RNA and DNA in the cells are in some way interrelated. This view does not conflict with their possible roles in protein synthesis, since all the evidence from studies with tracer elements indicates that the synthesis is accompanied by an intensification of the metabolic turnover of the nucleic acids rather than a process of transformation.

One puzzling aspect of the chemical composition of the fully grown explants remains to be discussed. It is generally accepted that the growth of cells *in vitro* is accompanied by a return to a more generalised cell type (21), or by a process of 'dedifferentiation' (9). In our studies on the chemical development of chick embryo organs (3), we found that differentiation was accompanied, amongst other things, by a striking increase in the protein content of the cells, and we suggested that this might be characteristic of cells which were developing to their adult or specialised forms. In a more recent paper we have reviewed studies on the chemical changes in induced tumours and found evidence to support the view that tumour cells contain much less protein than the corresponding cells in normal tissue (4). This, too, can be understood as part of the chemical change involved in the return from the normal adult cell to a more primitive form. But we do not find a similar change in the composition of the chick heart cells cultivated *in vitro*, as might be expected from the concept of their 'dedifferentiation.' It is quite clear from our present results that the final cell composition is not significantly different from that of the cells in the 12 day embryo heart, even after a seven-fold increase in their number *in vitro*, and that the decreasing amounts of protein per cell are unconnected with 'dedifferentiation' as we had previously suggested (3).

SUMMARY

1. Cell number and changing cell composition have been determined in chick heart explants from 12 day embryos during growth *in vitro*. The determination of cell number has been based on the deoxyribonucleic acid (DNA) content of the cultures, since it has been established that there is a constant amount of DNA phosphorus (DNAP) per nucleus of about 2.35×10^{-7} μg in all the somatic cells of the fowl. By reference to the DNAP of the cultures it has also been possible to express as amounts per cell the protein nitrogen (PN), ribonucleic acid phosphorus (RNAP), lipid phosphorus (LP), and acid-soluble phosphorus (ASP) of the cultures during the various phases of growth.

2. There was an initial fall of two-thirds in the number of cells per 48 explants when these were in contact with Tyrode-serum mixture during the 24 hours following planting. This initial loss in cell number could be reduced but not prevented by keeping the cells in growth-promoting medium from the time of planting. In both media there were similar alterations in cell composition, namely large increases in the amounts of PN and LP per cell.

3. After this initial stage in Tyrode-serum the cultures received fresh amounts of growth-promoting medium every 24 hours. Cell number increased most rapidly between 48 and 144 hours when the numbers per 48 explants doubled in each 48 hour interval. After 144 hours the increase was slower and linear until 240 hours.

4. Within certain limits the number of cells in the initial explants determined the final number in the fully grown cultures. The largest increases in cell number recorded in these experiments were between six and sevenfold.

5. Growth of the cultures was accompanied by a consistent pattern of changes in the chemical composition of the cells. The intensive cell division in the cultures was characterized by large increases in the PN and LP per cell in the first stage of culture growth, and by later increases in the RNAP and ASP per cell to maximal amounts during the period of rapid cell multiplication. The amounts of all four cell constituents (PN, RNAP, LP, and ASP) decreased to approximately their original amounts per cell in the chick heart cells when the culture growth slowed down after 144 hours.

6. These results suggest that the synthesis of protein and phospholipid

precede the synthesis of DNA in the chemical processes leading to cell division, and that RNA are in some way related to DNA synthesis.

7. It was found that keeping 48-hour growing cultures in Tyrode-serum for a further 24 hours resulted in a small increase in cell number, but produced little or no change in cell composition; it did not impair their ability to grow normally when returned to growth-promoting medium.

We wish to acknowledge with thanks that this work has been supported by a grant from the Medical Research Council to one of us (J. N. D.) and by a grant from the Rankin Fund of the University of Glasgow. We are grateful to Dr. Iwo Lominski and Dr. A. O'Hea of the Bacteriological Department, Western Infirmary, Glasgow for helpful advice on the aseptic technique employed in the tissue culture work, and to Dr. H. S. D. Garven of the Physiology Department for histological advice and help with photomicrography. Finally, we should also like to thank Miss M. Meikle for her valuable and careful technical assistance.

REFERENCES

1. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 286 (1938).
2. Brues, A. M., Rathbun, E. N., and Cohn, W. E., *J. Cellular Comp. Physiol.*, **24**, 155 (1944).
3. Davidson, J. N., and Leslie, I., *Nature*, **165**, 49 (1950).
4. —— *Cancer Research* **10**, 587 (1950).
5. Davidson, J. N., Leslie, I., Smellie, R. M. S., and Thomson, R. Y., *Biochem. J.*, **46**, proc. xl. (1950).
6. Davidson, J. N., Leslie, I., and Waymouth, C., *Biochem. J.*, **44**, 5 (1949).
7. Davidson, J. N., and Waymouth, C., *Biochem. J.*, **37**, 271 (1943).
8. Ephrussi, B., and Hugues, Y., *Compt. Rend. Soc. Biol.*, **105**, 697 (1930).
9. Fischer, A., Biology of Tissue Cells, Cambridge University Press, 1946.
10. Hull, W., and Kirk, P. L., *J. Gen. Physiol.*, **33**, 325 (1950).
11. —— *J. Gen. Physiol.*, **33**, 335 (1950).
12. —— *J. Gen. Physiol.*, **33**, 343 (1950).
13. Mirsky, A. E., and Ris, H., *Nature*, **163**, 666 (1949).
14. Ogur, M., and Rosen, G., *Arch. Biochem.*, **25**, 262 (1950).
15. Sanford, K. K., Earle, W. R., and Likely, G. D., *J. Natl. Cancer Inst.*, **9**, 229 (1948).
16. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
17. Signorotti, B., Hull, W., and Kirk, P. L., *J. Gen. Physiol.*, **33**, 315 (1950).
18. Tomkins, E. R., Cunningham, B., and Kirk, P. L., *J. Cellular Comp. Physiol.*, **30**, 1 (1947).
19. Vendrely, R., and Vendrely, C., *Experientia*, **4**, 434 (1948).
20. —— *Experientia*, **5**, 327 (1949).
21. Willmer, E. N. in "Essays in Growth and Form." Edited by W. E. Le Gros Clark and P. B. Medawar, Clarendon Press, Oxford, pp. 264–294 (1945).

THE NUCLEAR REDUCTION PROCESS IN BACTERIA

K. A. BISSET, JOYCE GRACE, and E. O. MORRIS

Department of Bacteriology, University of Birmingham, Great Britain

Received October 30, 1950

INTRODUCTION

One of the first, convincing descriptions of the process of nuclear reduction in bacteria was that of Klieneberger-Nobel (5), who showed that, in certain sporing bacilli, the formation of a rod-shaped fusion nucleus is followed by division of the nuclear material into four masses, one of which is included in the spore, the rest being rejected. It was subsequently shown by Bisset (1, 2), that this process is typical only of multicellular bacilli, and that in unicellular species only two nuclear units are formed, of which one is rejected. This scheme was verified by phase-contrast microscopy upon living material by Pulvertaft (10) (Plates I and II). Bisset (3) also demonstrated the essential similarity of the processes of maturation of the resting cell in various types of bacteria, and suggested that the evidence, both cytological and genetical, indicates that in each case the diploid generation is short-lived, and that reduction immediately precedes maturation, as with certain yeasts. A much earlier description by Lindegren and Mellon (8) of the nuclear cycle of *Mycobacterium tuberculosis* is also in general accordance with these conclusions.

In the course of investigation of these processes as they occur in *Bacterium aerogenes,* various *Myxobacteriales* and *Actinomyces bovis* it was found that nuclear reduction occurred, in each case, in a very similar manner, closely resembling that already described in sporing bacilli.

TECHNIQUE

Observations were made upon water-mounted material, stained by the Acid-Giemsa or methylene-blue-eosin methods, or by Giemsa alone. (See (3) for technique.)

OBSERVATIONS

Bacterium aerogenes. The final stages of the maturation of the microcyst are not well seen in most Gram-negative bacteria, because the microcyst itself is so small. In many strains of *Bact. aerogenes,* however, it could be

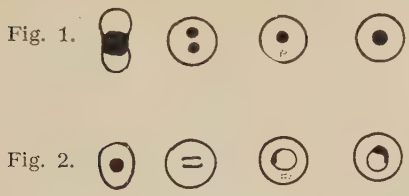


Fig. 1. Diagram of the reduction process as it occurs in *Bact. aerogenes* and in *Cytophaga sp.*, commencing at the stage on nuclear fusion.
Fig. 2. Diagram of the reduction process as it occurs in myxobacteria.

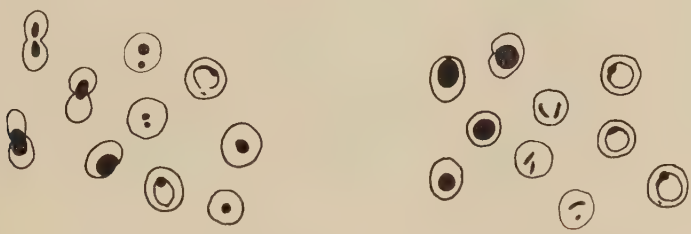


Fig. 3. Fig. 4.

Fig. 3. Stages in the maturation of the microcyst in *Cytophaga sp.*
Fig. 4. Stages in the reduction process in myxobacteria.

seen that, after the formation of the central nucleus, by a fusion process (1), a nuclear division took place. This division was unaccompanied by cell-division and was entirely distinct from that which precedes the formation of the fusion nucleus, at an earlier stage of the maturation process, and which takes place in an elongated instead of an almost spherical cell. The two daughter nuclei appeared in all stages between complete equality in size and almost complete degeneration of one at the expense of the other (Plates III & IV).

By analogy with the condition in the other groups recorded in this paper, in which a progressive morphological change accompanies a similar nuclear division, this phenomenon is interpreted as a reduction process (Text fig. 1).

Myxobacteriales. Several species of *Myxococcus* and *Chondrococcus*, and eleven strains of *Sporocytophaga myxococcoides* were examined. The first part of the cycle was similar to that described by Grace and quoted by Bisset (1, 3). The initial formation of a central nucleus, which took place in a rounded cell in myxobacteria (Text figs. 2, 4), and in a constricted cell in *Sporocytophaga* (Text figs. 1, 3) was succeeded by its division into two halves, one of which degenerated. In *Sporocytophaga*, as in *Bact. aerogenes*, these daughter nuclei were, apparently, small, spherical bodies (Plate 5;

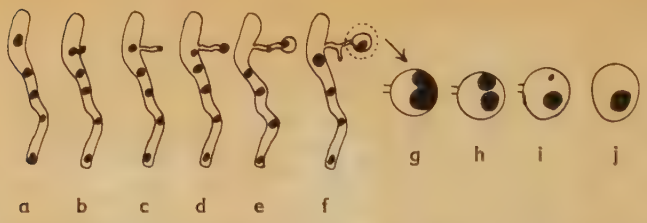


Fig. 5. Diagram of the reduction process as it occurs in *Actinomyces bovis*, commencing with the formation of the stalk of the microcyst on the parent filament.

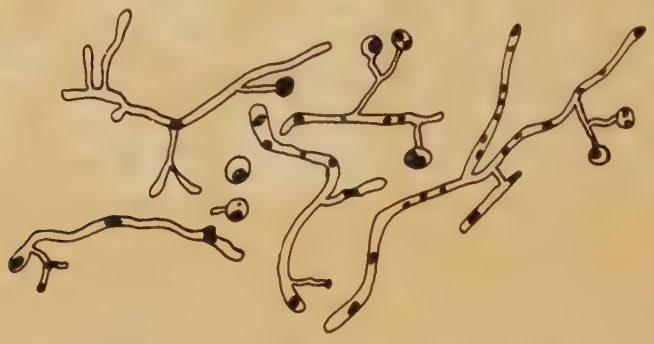
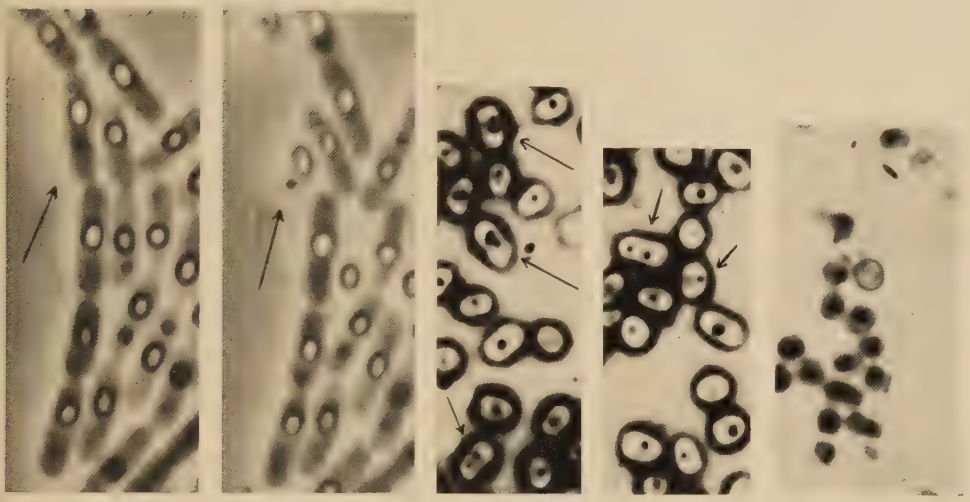


Fig. 6. Stages in the maturation of the microcyst in *Actinomyces bovis*.

Text figs. 1, 3); in myxobacteria they often took the form of rod-like chromosomes, reminiscent of those figured by Lindegren and Mellon (8) in *M. tuberculosis* (Text figs. 2, 4). This process was observed in all strains, if the cultural conditions were favourable for the formation of mature microcysts. The cells in which this nuclear division occurs are, apparently, older than those in which the central fusion nucleus is found. Their age could be judged from their morphology and staining reactions. In both myxobacteria and *Sporocytophaga*, it was observed that the cell wall of the cells undergoing reduction division was thicker and stained more deeply than those in which the central nucleus was found, and in *Sporocytophaga* the central nucleus was formed in a constricted cell, whereas reduction occurred after the cell had become spherical. This division, therefore, must succeed and cannot precede the formation of the central nucleus, and is regarded as reductional.

The outline of this process is recorded by Krzemieniewska (7) and Klieneberger-Nobel (5).

Actinomyces bovis. The complete life cycle of this organism has been recorded elsewhere (9). A haploid mycelium arises from the germination



I II III IV V

PLATES I and II

Stages in the maturation of the spore of a large *Bacillus*, photographed by phase-contrast. In Plate I the sporangium is still intact, one nuclear unit has been included in the spore, the other remains in the body of the cell. In Plate II the spore and the rejected nucleoid are released by the dissolution of the sporangium. × 2 500 approx. (By the courtesy of Prof. R. J. V. Pulvertaft.)

PLATES III and IV.

Stages in nuclear reduction in maturing microcysts of *Bact. aerogenes*. Several cells show division of the nucleus into two spherical masses, totally unlike the chromosomal division of the vegetative cell. The dark material is mucus. Stained by methylene-blue-eosin. × 3 000.

PLATE V

Mature microcysts and a reduction figure in *Cytophaga sp.* Stained by Giema. × 3 000.

of the spore; from this generation an initial cell is formed by fusion of two modified cells. The initial cell gives rise to the second, diploid generation, which is composed of coenocytic filaments, the nuclear material being arranged in a haphazard manner. When the diploid generation is well developed, after 4 -12 days, according to the strain and environment, spores begin to appear. A very slender tube grows out from the side of the filament, and a nuclear mass moves towards it. The nucleus divides into two equal parts, one part remains in the filament and the other part passes along the tube to the distal end (Text fig. 5, a, b, c, d).

The nucleus of the mature spore arises from this small nuclear mass at the end of the tube. At first there is no apparent cytoplasm. The nucleus grows to about twice the diameter of the spore tube, and the cytoplasm

begins to appear, after which the spore grows rapidly to form a cell 2 or 3 times the diameter of the diploid filament (Text fig. 5, e, f). When this stage is fully developed the nucleus is at least half the diameter of the cell. This nucleus divides into two equal parts, one of these remains unaltered, and the other becomes smaller and apparently less regular in form, and finally disappears (Text fig. 5, g, h, i, j). The final mature spore is a spherical cell with an eccentrically placed nucleus, and this is the typical picture of the resting microcyst of bacteria (4) (Text figs. 5, 6).

It is apparent from a study of the complete life cycle of this organism that the appearances described above represent a nuclear reduction process.

SUMMARY AND CONCLUSIONS

The reduction process in bacteria belonging to several major groups, sporing and non-sporing eubacteria, myxobacteria, cytophagas and actinomyces, occurs in a very similar manner. It immediately precedes the maturation of the resting or distributive stage, and takes the form of a nuclear division, unaccompanied by cell division. Of the two daughter nuclei, one is transformed into a vesicular, resting nucleus, the other degenerates.

REFERENCES

1. Bisset, K. A., *J. Hyg.*, **47**, 182 (1949).
2. —— *J. Gen. Microbiol.*, **4**, 1 (1950).
3. —— The Cytology and Life-History of Bacteria, Edinburgh, 1950.
4. —— *Exptl. Cell. Res.*, **1**, 473 (1950).
5. Klieneberger-Nobel, E., *J. Hyg.*, **44**, 99 (1945).
6. —— *J. Gen. Microbiol.*, **1**, 33 (1947).
7. Krzemieniewska, H., *Acta Soc. Bot. Pol.*, **7**, 507 (1930).
8. Lindegren, C. C., and Mellon, R. R., *Proc. Soc. Exptl. Biol. Med.*, **30**, 110 (1933).
9. Morris, E. O., *J. Hyg.* (in Press) (1951).
10. Pulvertaft, R. J. V., *J. Gen. Microbiol.*, **4**, xiv (1950).

THE EGGS AND HALF-EGGS OF ARBACIA PUNCTULATA AND THE PLUTEI, AS PHOTOGRAPHED BY ULTRAVIOLET, VISIBLE AND INFRARED LIGHT

E. B. HARVEY and G. I. LAVIN

Marine Biological Laboratory, Woods Hole, Mass., Biological Laboratory, Princeton University, N. J., and Rockefeller Institute for Medical Research, New York City

Received November 9, 1951

EGGS

When an attempt is made to photograph the intact normal or centrifuged eggs of *Arbacia punctulata* with ultraviolet light (Figs. 5, 6, 7) not much structure is to be seen, not as much as with visible light (Figs. 1, 2). They are apparently so thick that under present experimental conditions almost complete absorption of the radiation (2,537Å) takes place. The normal egg has a diameter of 74 μ.

The nucleus is seen as a clear body; this one would not expect if it were made up uniformly of nucleic acid compounds. It is known that the chromatin material in the resting nucleus is in the form of a reticulum, lying in a clear nucleoplasm. In a previous paper (4) it was shown that this reticulum absorbs in the ultraviolet and that it also takes chromatin stains (see enlarged Figs. 4–6 in paper just mentioned). The greater part of the nucleus is non-absorbing and appears light, not dark when photographed with ultraviolet light.

When the normal (Fig. 8) or the centrifuged egg (Figs. 9, 10) is slightly compressed and thus made thinner, enough light gets through and the cytoplasmic structure can be observed. The centrifuged egg breaks at the clear layer and this appears granular; the granules absorb ultraviolet light. The granular structure of this layer is also apparent in fixed preparations stained with haematoxylin, but is not marked in fixed (with formalin) preparations, unstained, as photographed with ultraviolet light ((4) figs. 11, 12 and 13). It must be the granules imbedded in the matrix and not the liquid matrix itself which contains the nucleic acid compounds, since it is these granules which, in the living centrifuged egg, absorb the ultraviolet, as shown in Figs. 9, 10. The yolk and pigment are also absorbing; the oil cap absorbs strongly.

With further centrifugation the egg breaks into two nearly equal "halves," the white and red (1, 2). The granular structure of the white half is well shown by ultraviolet light especially when it is slightly squashed (Figs. 11, 12). It is different from the visible light picture (Fig. 3) in that the clear layer now appears granular, as in the whole egg, as noted above; the mitochondrial layer is not distinct. The granules are also observed when stained with haematoxylin (3). The red half shows the absorbing nature of the yolk and, in a greater degree, of the pigment. The photograph of the red half with ultraviolet light (Fig. 13) is similar to that obtained with visible light (Fig. 4).

In the previous paper (4) a photograph (Fig. 9) showed the absorption of ultraviolet light by the chromosomes of a normal dividing egg. These are not apparent with visible light (Fig. 7 in the paper referred to).

With infrared the centrifuged eggs appear much as they do when photographed with visible light. However, the red pigment granules are not black and appear not to be distinguished from the yolk. The yolk granules often appear more coarse than when photographed with visible light (Figs. 14, 15). The mitochondrial layer shows well in some photographs (Fig. 14).

PLUTEI

The normal pluteus two days after fertilization is shown as photographed by visible light on Plate II, Figs. 1 and 2. The most obvious structures are the four arms with skeletal rods, the digestive tract consisting of a large mouth, oesophagus and stomach. The pigment spots are irregularly arranged through the body, especially along the arms and at their tips.

When photographed with ultraviolet light the oesophagus and stomach appear dark as do also the two transverse ciliated bands, the oral band around the mouth and the post-oral (or ventral) band between the two long (anal) arms above the anal opening (Figs. 3, 4). The pigment spots and skeleton appear as with visible light. There may be some correlation be-

---

PLATE I

Figures 1–4. Normal whole egg (1), centrifuged egg (2), and white (3) and red (4) "halves," photographed with visible light.

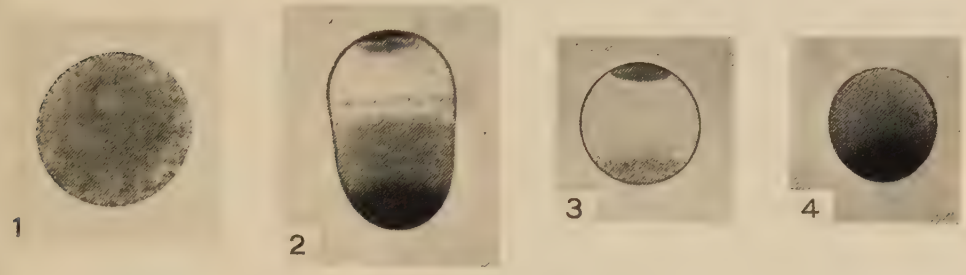
Figures 5–7. Normal whole egg (5), normal two-cell stage (6), and centrifuged egg (7), intact, photographed with ultraviolet.

Figures 8–13. Normal whole egg (8), centrifuged egg (9, 10), and white (11, 12) and red (13) "halves," all slightly squashed, photographed with ultraviolet.

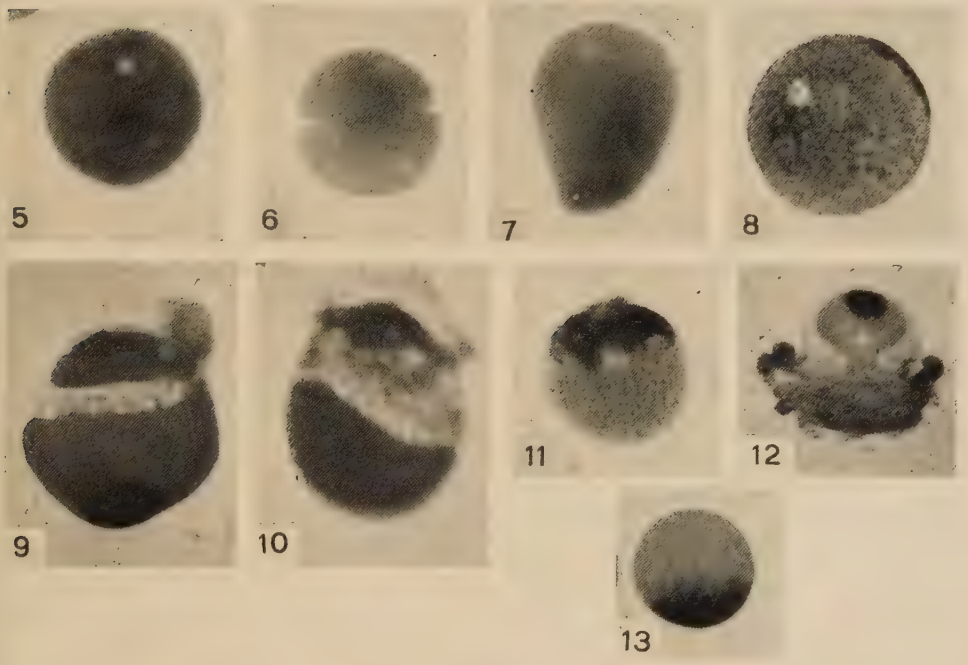
Figures 14, 15. Centrifuged egg photographed with infrared.

All figures in Plate I enlarged about 300 ×.

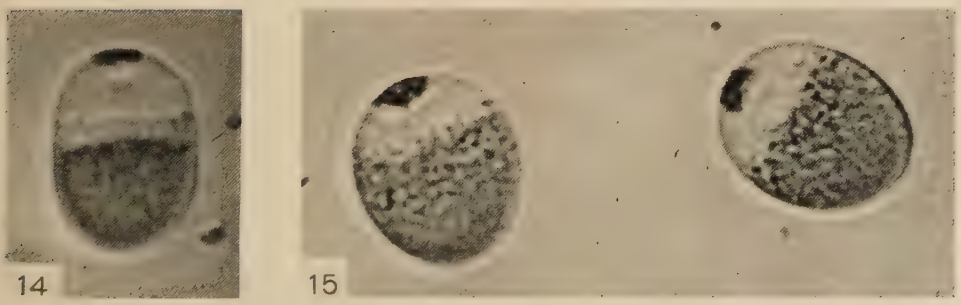
Visible



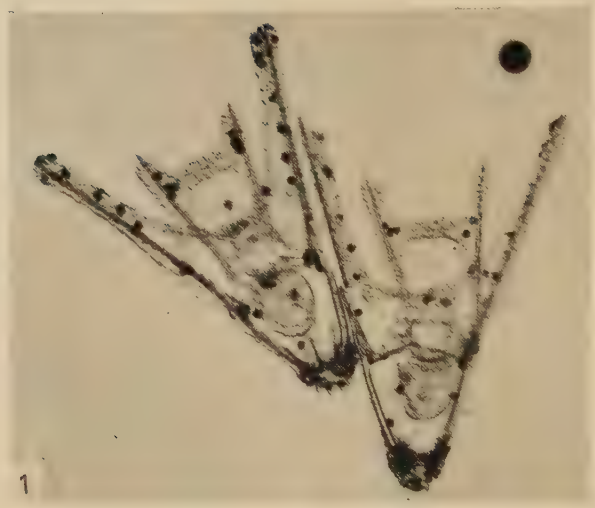
Ultraviolet



Infrared



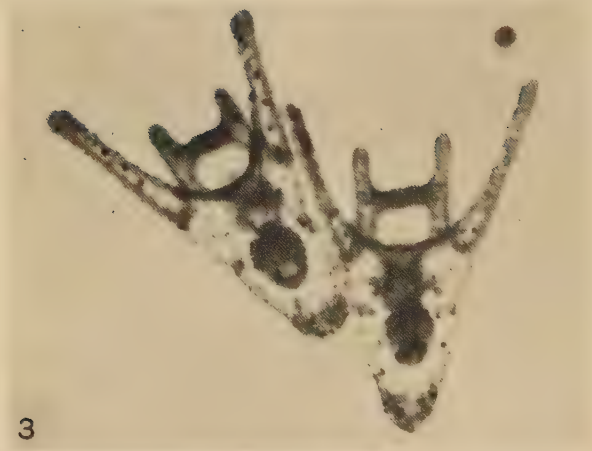
Visible



1

2

Ultraviolet



3

4

Infrared

5

6

tween the ultraviolet absorbing regions (alimentary canal and transverse ciliated bands) and physiological activity (digestion, procuring of food and swimming. When fixed preparations are stained with haemotoxylin, these same regions, the alimentary canal and two transverse ciliated bands, are deeply stained.

When photographed with infrared the structures appear much as they do with visible light except that the red pigment spots are not distinguishable (Figs. 5, 6).

TECHNIQUE

The ultraviolet photomicrographs were obtained by the technique described by Lavin (5). A resonance radiation mercury vapor lamp is the light source (about 80 per cent of the energy emitted is in the form of the 2,537Å line). The visible and other lines are filtered out by means of an aqueous solution of cobalt sulfate and nickel sulfate. An image of the material under examination is focussed on a screen (willemite) and a field selected. A photographic plate is then substituted for the screen and photographed in the usual manner.

The plutei shown on Plate II, Figs. 1 and 2, were taken (by Lavin) with the 5,460Å mercury line from the same lamp used for the ultraviolet. This line was isolated by means of a Wratten filter No. 77A. The visible light photographs on Plate I were taken (by Harvey) with the usual light source.

For the infrared a Wratten filter No. 25A was used (by Lavin) to focus, after which it was replaced by an 88A filter. Metallographic plates were used for the ultraviolet, Kodak M plates for the visible, and infrared sensitive plates for this region. (8 000–10 000Å.)

SUMMARY

Normal and centrifuged eggs, the halves obtained by strong centrifugal force and the plutei of *Arbacia* have been photographed with visible, ultraviolet (1,537Å) and infrared (8,000–10,000Å) light. Differences in appearance with these different light sources are described and some of the photographs reproduced.

REFERENCES

1. Harvey, E. B., *Biol. Bull.*, **62**, 155 (1932).
2. —— *Biol. Bull.*, **79**, 166 (1940).
3. —— *J. Exptl. Zool.*, **102**, 253 (1946).
4. Harvey, E. B., and Lavin, G. I., *Biol. Bull.*, **86**, 163 (1944).
5. Lavin, G. I., *Rev. Sci. Instr.*, **14**, 375 (1943).

Plate II

Figures 1–6. Two day plutei photographed with visible (1, 2), ultraviolet (3, 4), and infrared (5, 6) light. Figures 1 and 3 are of the same plutei, with light source changed. 175 ×.

NUCLEI OF ARBACIA AND CHAETOPTERUS EGGS AS PHOTOGRAPHED BY INFRARED LIGHT

E. B. HARVEY and G. I. LAVIN

Marine Biological Laboratory, Woods Hole, Mass., Biological Laboratory, Princeton University, N. J., and Rockefeller Institute for Medical Research, New York City

Received December 1, 1950

It has been found previously by these writers that the red pigment granules in the *Arbacia* egg and pluteus do not show in photographs taken with infrared light. It was therefore thought that cleavage figures photographed with infrared light might be of interest. Such proved to be the case.

With visible light (Figs. 1, 3, 5, 7), the red pigment obscures the nuclei and division figures, but when photographed with infrared light, these stand out with remarkable clearness as white areas against the dark background of the cytoplasm. The streak stage of the early prophase (Fig. 2), the dumb-bell shaped mitotic figure of the metaphase, the separated spheres of the telophase, and the two nuclei and division plane following first cleavage are clearly shown (Fig. 4). Likewise one may clearly see the mitotic figures and division planes of subsequent cleavages (Fig. 6). In the early and late blastulae, the nuclei stand out with great clearness; in fact one can actually count the numbers of nuclei present in an optical section at various stages and calculate the numbers present in the whole egg (Fig. 8). This method might prove of value in relating chemical changes in the developing egg with progressive cleavages.

The nuclei in the red half egg which are present after fertilization of the non-nucleate half (1, 2, 4) can be seen much more clearly than with visible light, both as a single nucleus soon after fertilization and as two or more nuclei after its division (Figs. 9, 10, 11). It will be of interest to study photographs of the parthenogenetic merogones (developing non-nucleate parthenogenetic half-eggs) with infrared (2, 4).

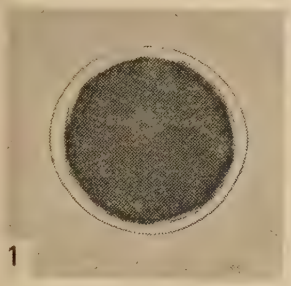
Plate I (Magnif. 280 ×).

Fig. 1. *Arbacia punctulata*, streak stage, 30 minutes after fertilization, visible light.
Fig. 2. Same, infrared light.
Fig. 3. Spindle stage and just after cleavage, 45–60 minutes after fertilization, visible light.
Fig. 4. Same, infrared light.

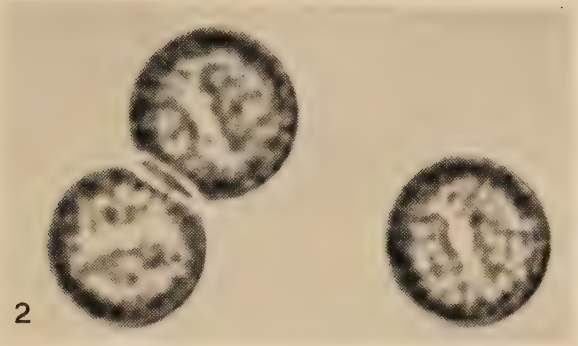
Visible

ARBACIA

Infrared



1



2

3

4

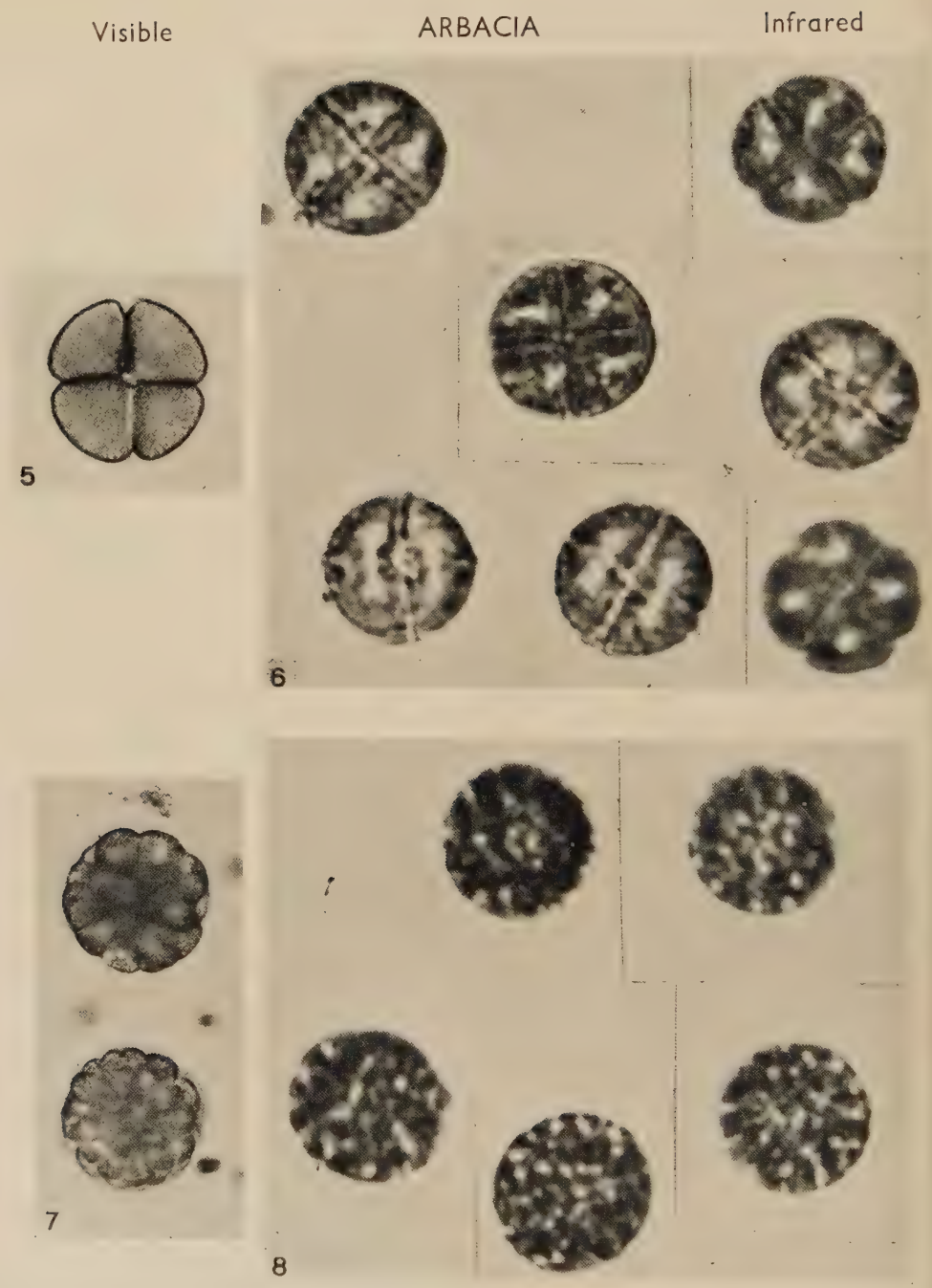


Plate II (Magnif. 280 ×).

Fig. 5. *Arbacia punctulata*, 4-cell stage, 1 $^1/_2$ hrs. after fertilization, visible light.
Fig. 6. Same, infrared light.
Fig. 7. Blastulae, 3–4 hrs. after fertilization, visible light.
Fig. 8. Early and late blastulae, 3–8 hrs. after fertilization, infrared light.

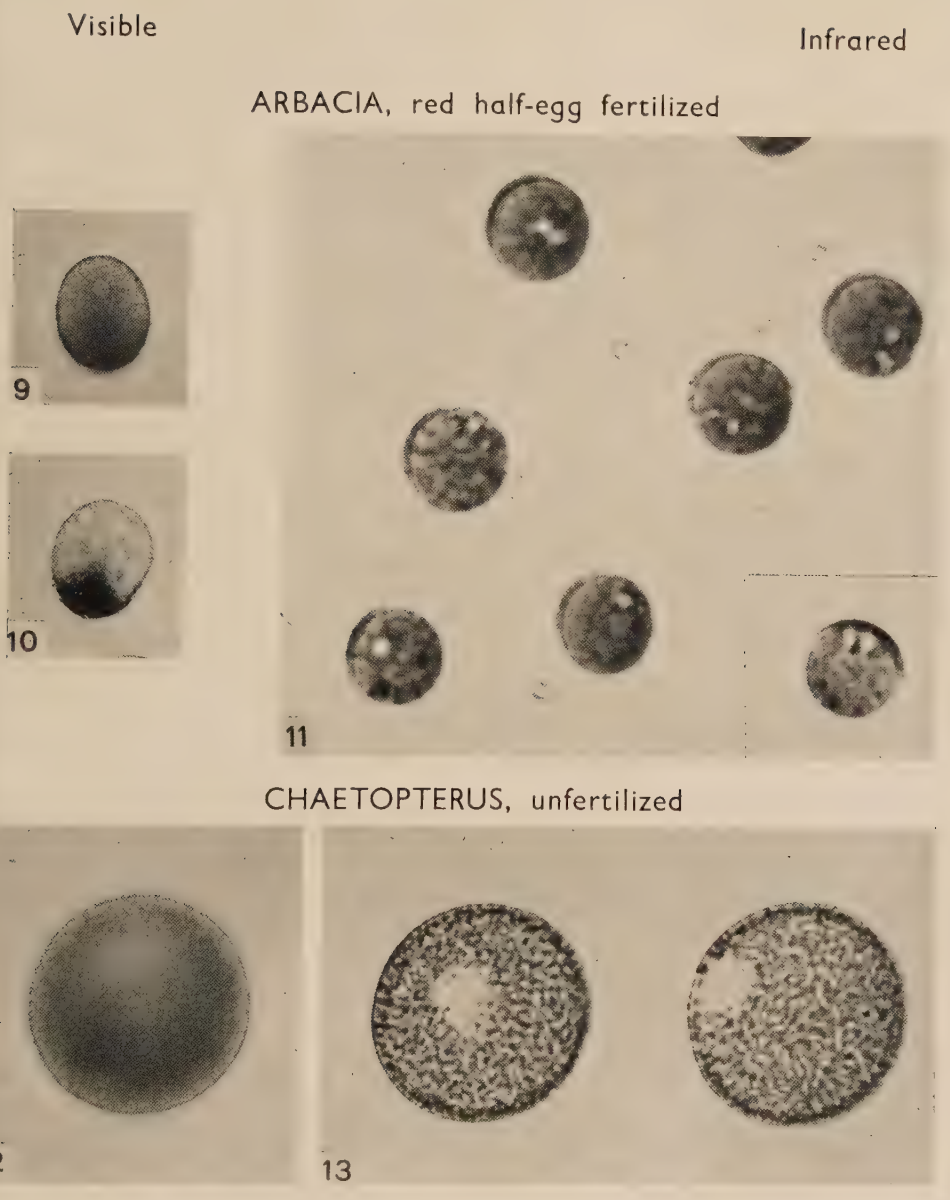


Plate III.

Figs. 9–11, magnif. 250×; Figs. 12, 13, magnif. 300×.
Fig. 9. *Arbacia punctulata*, red half, 20 minutes after fertilization, visible light.
Fig. 10. Same 2 hrs. after fertilization.
Fig. 11. *Arbacia punctulata*, red half, 20 minutes–2 hrs. after fertilization, infrared light.
Fig. 12. *Chaetopterus pergamentaceus*, unfertilized, visible light.
Fig. 13. Same, infrared light.

The *Chaetopterus* egg, when viewed or photographed with ordinary light (Fig. 12) is quite opaque, and not much structure can be seen (3). In the infrared photographs the granular structure of the cytoplasm is seen, and the nuclear material of the unfertilized egg appears as a brilliant white area (Fig. 13).

The general configuration of mitotic figures and nuclei is brought out by infrared because of its great penetrating power, especially through the red pigment granules. The detailed structure of the spindle and asters is not brought out by infrared because of its relatively poor resolving power. It is also owing to this that we do not see the fine granules in the cytoplasm, but only the coarser granules, as is evident in most of the photographs.

TECHNIQUE

The eggs were photographed by the use of Kodak Infrared Sensitive plates. The developer was D 19. A tungsten microscope lamp was the light source. An image was arranged and focussed with a deep red light filter (Wratten 24 A) after which an infrared transmitting light filter (Wratten 88 A) was substituted for the photography.

SUMMARY

Photographs, taken with infrared light, of all stages of dividing *Arbacia punctulata* eggs show the configuration of mitotic figures and nuclei as brilliant white areas against the dark granular cytoplasmic background. The photographs are very different from those taken with visible light. The nucleus and cytoplasm of the unfertilized *Chaetopterus pergamentaceus* egg which are quite opaque with visible light are quite striking with infrared light.

REFERENCES

1. Harvey, E. B., *Biol. Bull.*, **62**, 155 (1932).
2. —— *Biol. Bull.*, **71**, 101 (1936).
3. —— *Biol. Bull.*, **76**, 384 (1939).
4. —— *Biol. Bull.*, **79**, 166 (1940).

PERIMENTAL CELL RESEARCH

CORRECTION

Vol. II, No. 3, p. 402, line 14: (Wratten 24 A) *read* (Wratten 25 A).

THE ACTION OF CERTAIN POLYSACCHARIDES ON FERTILIZATION IN THE SEA URCHIN EGG

C. V. HARDING[1]

Marine Biological Laboratory, Woods Hole, Mass., and Zoological Laboratory, University of Pennsylvania, Philadelphia, Pa.

Received November 21, 1950

According to the concept developed by Runnström (14), the fertilization membrane is formed from the vitelline membrane, which covers the surface of the unfertilized egg, and material exuded from the cortex of the egg at the time of fertilization. Observations on sea urchin eggs at various stages of ripeness, and their capacity to elevate normal fertilization membranes, has led to the hypothesis that an inhibitor to activation is present in the unfertilized egg. There are indications that this inhibitor may be a polysaccharide (16).

In accordance with the above hypothesis, it has been found that complete differentiation of the fertilization membrane is prevented when eggs are fertilized in the presence of certain polysaccharides. For example, normal elevation of the fertilization membrane is inhibited when the eggs of *Echinocardium cordatum* are fertilized in the presence of a 0.025 per cent solution of jelly coat substance (15). The cortical material does not fuse in normal fashion with the vitelline membrane, and a low, thin fertilization membrane is formed instead of the normal, well-elevated, thick membrane. Heilbrunn and Wilson (5) have found that heparin inhibits fertilization in eggs of *Chaetopterus*, and also prevents cleavage. Runnström and Wicklund (18) demonstrated with the eggs of *Strongylocentrotus droebachiensis* and *Arbacia lixula* that heparin (from the Vitrum Co.) acts in a manner similar to that of the jelly coat substance, only in higher concentrations.

The present experiments were designed to obtain information about the comparative actions of three samples of heparin and two other polysaccharides on the fertilization process in the eggs of *Arbacia punctulata* and *Echinarachnius parma;* and the effect of sodium periodate oxidation on the activity of these polysaccharides. It was planned also to obtain some information about the site of action, as well as a possible mechanism of action of the polysaccharides.

[1] National Institutes of Health Postdoctorate Fellow.

METHODS AND MATERIALS

Eggs were obtained from *Arbacia punctulata* by inducing shedding. This was accomplished by injecting a few drops of isotonic KCl into the oral opening. In a few cases, injections were made into the individual gonopores. The eggs were washed with filtered sea water and strained through cheese cloth twice to remove bits of tissue, and then kept at the temperature of running sea water (approx. 22° C) until used. Sperm were obtained by injecting a few drops of isotonic KCl into the oral opening. The "dry sperm," obtained in this way, were used immediately or stored at about 7° C until used. Ordinarily, sperm were not kept more than two days before being used. For fertilization, a diluted sperm suspension was prepared by adding one drop of "dry sperm" to 5 ml of filtered sea water. One drop of this was added to 2 ml of sea water containing the eggs.

To induce shedding in *Echinarachnius parma* a small portion of the oral surface was removed and several drops of isotonic KCl were then placed in the body cavity. The animal was then placed, oral surface up, on a stender dish, filled with sea water. The eggs, obtained in this way, were washed with filtered sea water and strained through cheese cloth twice. In some cases green pigment from the animal escaped into the sea water containing the eggs. In such cases the eggs were discarded. Sperm were obtained by removing the testes from the animal. Dry sperm, shed from the isolated testes, were taken up in a fine pipette and transferred to a small test tube. For fertilization, sperm dilutions were made as in the case of the experiments with *Arbacia.*

The sea water used in all experiments was filtered, and all solutions, unless otherwise stated, were made up in filtered sea water. The samples of heparin used were obtained from the following sources: Hynson, Westcott, and Dunning Co., Na salt, lot number 211, 60 Int. Units per mg; Upjohn Co., Na salt, lot 257-LLÇ-2, 100 Int. Units per mg (obtained through the kindness of Mr. Norman Anderson); Vitrum Co., Na salt, 80 Int. Units per mg. The sample of "fucoidin" was prepared from the plant *Fucus vesiculosis* by Vasseur. The sample contained other polysaccharides besides fucoidin. Paper partition chromatography of the hydrolysate showed a large spot for glucose, smaller spots for fucose, xylose, and mannose, and a very small spot for galactose. A mucopolysaccharide, identical with the blood group H substance, extracted from pig mucin was made available to Professor John Runnström by Dr. W. T. J. Morgan of the Lister Institute.

The pH of each solution did not vary appreciably from 7.8. In some experiments, 0.05 per cent solutions of Vitrum heparin and also Upjohn heparin, made up in sea water, were heated in an autoclave at 119° C for from 10 minutes to 30 minutes. Measurements of the pH were made before and after heating. If necessary, the pH was adjusted to approximately 7.8.

A stock solution of 0.1 N $NaIO_4$ was prepared in distilled water. For each experiment, a small volume of the stock solution was diluted 100 times with unbuffered artificial sea water. A 0.1 or 0.2 ml sample of this 0.001 N sodium periodate was then added to 2 ml of the test solution. Therefore, the final concentration of periodate was 5 to 10×10^{-5} N. This procedure is adapted from Runnström and Kriszat (16).

Crystalline trypsin was obtained from Armour Co. A 0.1 per cent solution was used in all experiments. Since approximately 50 per cent of the Armour preparation is $MgSO_4$, the actual concentration of trypsin was about 0.05 per cent.

RESULTS

Arbacia eggs were placed in solutions of each of the substances listed in Table I. Ten minutes afterwards, the eggs were fertilized in the solutions. Observations were then made on the elevation of the fertilization membrane, and cleavage.

All preparations, under one condition or another, inhibited elevation of the fertilization membrane. The activity of the solution, however, depended upon the preparation of polysaccharide used. For example, of the three samples of heparin used, the Hynson, Westcott and Dunning preparation (H.W.D.) proved the most active in preventing elevation of the fertilization membrane. The samples of Vitrum heparin and Upjohn heparin were the least active of the five polysaccharides and did not inhibit fertilization membrane elevation in the concentrations used. However, if the 0.05 per cent solutions of these heparin samples were heated at 119° C. in an autoclave for 10 to 30 minutes, they became active in preventing fertilization membrane elevation, even when diluted to concentration of 0.005 per cent to 0.01 per cent. Eggs fertilized in these solutions elevated very thin membranes which were lifted only slightly above the egg surface. Within 2 to 3 minutes after fertilization, many granules became visible between the egg surface and the membrane. Such a distinct layer of granules could not be seen in those eggs treated with H.W.D. heparin. A possible explanation of this effect of heating is presented in the discussion. "Fucoidin," a polysacchride preparation extracted from *Fucus vesiculosis*, and the human H substance, a mucopolysaccharide, were also found to inhibit elevation of the fertilization membrane. Table I summarizes the results when the eggs of *Arbacia punctulata* were fertilized in various concentrations of these inhibiting substances.

In relatively low concentrations the H.W.D. heparin resulted in partial membrane elevation at localized areas of the egg surface. This was quite noticeable with *Echinarachnius parma* eggs, fertilized in 0.025 per cent H.W.D. heparin. The locally elevated areas of membrane appeared as blisters. Almost invariably, that part of the cortex, just below an *elevated* portion of the membrane, appeared more contracted than the rest of the cortex (see Figs. 1 and 2). This is similar to the action of jelly coat substance on *Psammechinus miliaris* eggs as reported by Runnström and Kriszat (16). Slightly higher concentrations of H.W.D. caused inhibition of membrane

TABLE I

Action of the polysaccharides, at various concentrations, on elevation of the fertilization membrane in the eggs of *Arbacia punctulata*.

Polysaccharide	Concentration				
	0.005 %	0.010 %	0.025 %	0.050 %	0.200 %
H.W.D.		no fert. membs. elevated	no fert. membs. elevated	no fert. membs. elevated	no fert. membs. elevated no cleavage
Vitrum			normal fert. membs.	normal fert. membs.	
Upjohn			normal fert. membs.	normal fert. membs.	
"Fucoidin"		no fert. membs. elevated	no fert. membs. elevated, no cleavage	no fert. membs. elevated, no cleavage	
H-subs.				(0.052 %) no fert. membs. elevated	
Autocl. Vitrum	fert. membs. partially elevated	no fert. membs. elevated	no fert. membs. elevated	no fert. membs. elevated	
Autocl. Upjohn			no fert. membs. elevated	no fert. membs. elevated	

elevation over the entire egg surface. *Arbacia* eggs appeared to be more sensitive than *Echinarachnius* eggs and concentrations lower than 0.025 per cent H.W.D. were required to cause inhibition over the entire egg surface.

In the low concentrations of H.W.D. heparin used in these experiments (approximate range of 0.01 per cent to 0.05 per cent) membrane elevation is inhibited, but, nevertheless, fertilization is accomplished and the eggs divide. Higher concentrations of heparin (0.2 per cent H.W.D.) more completely prevent cortical reactions in the *Arbacia* egg and cleavage does not occur. Thus, the action of H.W.D. heparin may be arbitrarily considered as two-fold. Each of these actions will be considered below.

Several questions arose concerning the nature of the mechanism of ac-

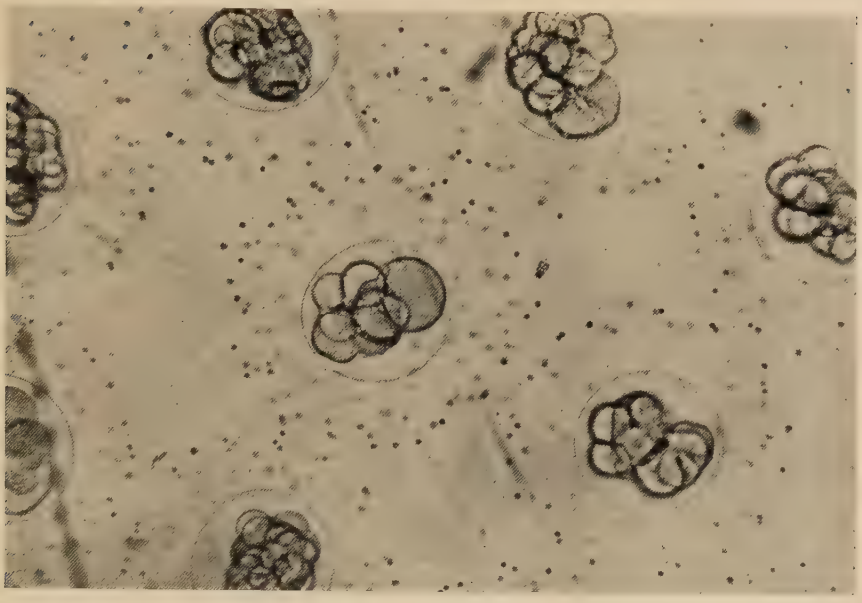


Fig. 1.

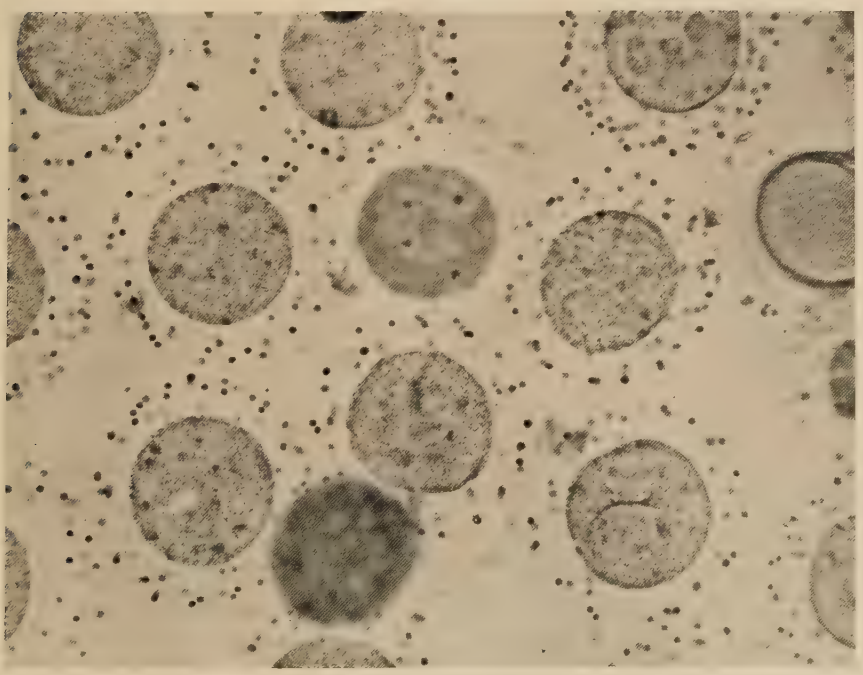


Fig. 2.

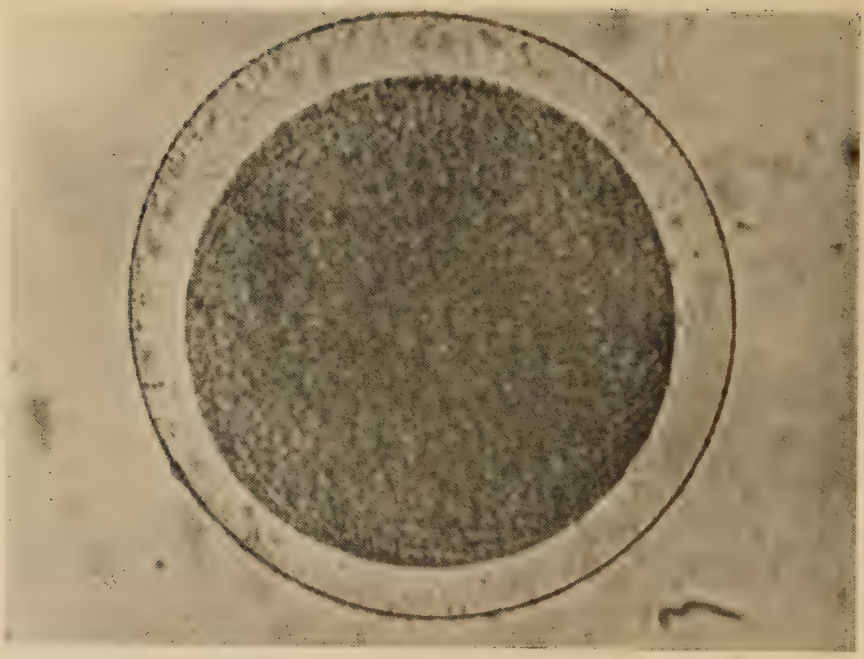


Fig. 3.

TABLE II

The Inactivation of Heparin[1] by $NaIO_4$.

Experiment number	% cleavage of eggs fert. in 0.2 % hep.	% cleavage of eggs fert. in 0.2 % hep. plus $NaIO_4$	Control
1	8	97	99
2	7	97	—
3	1	98	94
4	0	96	—

tion of the polysaccharides: 1) Is the effect due to the polysaccharide nature of the molecule or to some nonspecific effect? 2) Is the prevention of cleavage by heparin due to a blocking of fertilization or an inhibition of some phase of the cleavage cycle? 3) Is the action primarily on the egg or on the sperm? What, more exactly, is the site of action? 4) What possible chemical mechanism is involved? In an attempt to answer these questions, experiments were carried out with *Arbacia* eggs and H.W.D. heparin.

[1] Hynson, Westcott and Dunning.

Removal of Inhibitory Action of Heparin by Periodate Oxidation

Experiments were carried out which show that the oxidation of heparin with small amounts of sodium periodate removes its inhibitor action upon fertilization. This would indicate that the polysaccharide itself is the active inhibitory factor, and not some non-specific factor.

In one of several experiments, *Echinarachnius parma* eggs were divided into three batches. The first batch was fertilized in sea water, the second in 0.025 per cent H.W.D., and the third in 0.025 per cent H.W.D. heparin, containing a final concentration of 5×10^{-5} N $NaIO_4$. The $NaIO_4$ was added to the heparin solution 10 minutes before the eggs were added to the solution; the eggs were fertilized 10 minutes after being added to the solutions. Fertilization membrane elevation was inhibited in the 0.025 per cent H.W.D. (see Figs. 1 and 2). Eggs fertilized in the heparin-periodate mixture, however, showed fertilization membrane elevation. Some of the eggs fertilized in the heparin-periodate mixture represented intermediate stages of inhibition. For example, Fig. 3 is a photograph of an *Echinarachnius* egg 14 minutes after fertilization in the heparin-periodate mixture. The membrane is not as well elevated as in control eggs, and cortical granular material is visible in the perivitelline space and attached to the membrane.

Higher concentrations of H.W.D., which inhibit cleavage, were also inactivated with sodium periodate. *Arbacia* eggs were fertilized in 0.2 per cent H.W.D. and also in a mixture of 0.2 per cent heparin plus $NaIO_4$ to make a final concentration of 10^{-5} N $NaIO_4$. The number of dividing eggs was noted in each case. The results of four experiments are summarized in Table II. Here again, the inhibitory action of H.W.D. was removed with periodate oxidation.

Furthermore, the periodate oxidation of autoclaved solutions of Vitrum heparin and Upjohn heparin removed their inhibitory activity. Similar results were obtained by the periodate oxidation of solutions of 0.01 per cent fucoidin and 0.052 per cent H substance.

Inhibition of Cleavage Due to Inhibition of Fertilization

It seems most likely that the observed inhibition of cleavage by heparin is due to a prevention of normal fertilization. When sperm were added to *Arbacia* eggs in 0.2 per cent H.W.D. heparin there were no indications of fertilization membrane elevation and the eggs did not cleave. However, when eggs were fertilized in sea water and then transferred to 0.2 per cent

TABLE III

Effect of heparin on the cleavage of *Arbacia punctulata* eggs. The figures represent the percent cleavage of eggs treated in the manner indicated at the top of each column: 1) fertilized in sea water (control); 2) treated with 0.05 per cent trypsin for 30 minutes, transferred to sea water and fertilized; 3) treated with 0.05 per cent trypsin for 30 minutes, transferred to heparin solution and fertilized; 4) fertilized in heparin solution; 5) fertilized in sea water and then placed in heparin solution three minutes after fertilization; 6) Treated with 0.05 per cent trypsin for 30 minutes, transferred to sea water and fertilized, placed in heparin solution made up in calcium-free artificial sea water three minutes after fertilization; 7) treated with 0.05 per cent trypsin for 30 minutes, transferred to sea water and fertilized, placed in heparin solution made up in calcium-free artificial sea water five minutes after fertilization.

Per cent heparin	1	2	3	4	5	6	7
	Sea water control	Trypsin sea water fert.	Trypsin heparin fert.	Heparin fert.	Sea water fert. heparin	Trypsin sea water fert. heparin in Ca-free-3	Trypsin sea water fert. heparin in Ca-free-5
	Per cent Cleavage						
0.05 %			0	47	99	86	
0.10 %	95	97	0	45	94	92	
0.20 %	95	83		0	97	91	80
0.20 %						93	
0.20 %	95	90	0	0	100	97	95
0.20 %	93	91	0	0	95	93	95
0.20 %	99	99	0	0	99	96	99
0.20 %	66	89	0	0	95	71	79
0.20 %	99	98	0	0	92	91	

H.W.D. three minutes after fertilization, the eggs divided normally. Similar results have been obtained by Gagnon (3), working with Mactra eggs. It seemed possible that the fertilization membrane and hyaline layer, present in the fertilized egg, might prevent the heparin from reaching the egg surface. In order to facilitate penetration of the heparin to the egg surface, the fertilization membrane and hyaline layer were removed. This was done by treating the unfertilized eggs with a solution of 0.05 per cent trypsin in sea water for thirty minutes to dissolve the vitelline membrane (13). The eggs were then fertilized in sea water and no fertilization membranes were formed. Three minutes after fertilization they were transferred to 0.2 per cent heparin made up in artificial calcium-free sea water. The calcium-free sea water dissolved the hyaline layer (6). However, in this case also, high percentages of cleavage were obtained. Table III summarizes the results

of these experiments. It seems likely, therefore, that the eggs are most sensitive to heparin at the time of fertilization.

Eggs which were mixed with sperm in the presence of 0.2 per cent H.W.D. heparin were fixed 3 minutes, three hours and five hours later in Kahle's fixative. Sections of these eggs were stained with hemalum. There was no indication of a sperm pronucleus in any of the eggs. Sperm were seen surrounding the eggs, but no clearcut evidence of sperm penetration was found. If penetration did occur, no apparent further development took place.

Heparin Acts Upon Protoplasmic Surface of Egg

With regard to the third question, there is good evidence that the effect of the heparin is primarily on the egg rather than on the sperm. Eggs which were artificially activated reacted to heparin in a manner similar to eggs *fertilized* in the presence of heparin. *Arbacia* eggs which were exposed to hypertonic sea water (23 parts 2.5 N NaCl and 77 parts sea water) were induced to form "activation" membranes. However, eggs exposed to the same concentration of sea water, containing some H.W.D. heparin (final conc., 0.025 per cent) did not form "activation" membranes. This suggests that the heparin has its inhibitory action on fertilization primarily on the egg, and not the sperm.

Furthermore, it might be pointed out (as is evident from Table III) that heparin also inhibits fertilization in eggs from which the vitelline membranes have been removed by trypsin treatment. This eliminates the possibility that heparin inhibits fertilization by some direct action on the vitelline membrane. It was also found that heparin inhibits fertilization in *Arbacia* eggs from which the jelly coats had been removed mechanically by squirting them in and out of a narrow pipette. In control experiments, it was found that eggs, from which the jelly coats had been removed, formed normal fertilization membranes when fertilized in sea water. These results may be interpreted as an indication that the heparin molecule has its effect directly upon the protoplasmic cortex, and not indirectly, through some intermediate reaction with the jelly coat substance or the vitelline membrane.

DISCUSSION

Apparently, a variety of polysaccharides exert an inhibitory action on fertilization. The fact that very dilute solutions of sodium periodate remove

the activity of the heparin indicates that the polysaccharide itself is of importance in the inhibitory action, and not some extraneous factor.

The experiments reported above make it seem unlikely that heparin has its inhibitory effect on fertilization primarily through an action on the sperm, the jelly coat layer, or the vitelline membrane. The heparin probably has a direct action upon the protoplasmic surface of the egg. Assuming this to be true, we have a possible explanation of the paradoxical differences in activity of the three heparin samples used. Although H.W.D. heparin is the least active in terms of the prevention of blood clotting (60 Int. Units per mg, as compared with 80 and 100 for Vitrum and Upjohn, respectively), it is the most active in preventing fertilization. Such a situation might arise if it were possible for the H.W.D. molecules to penetrate the vitelline membrane, whereas the molecules of Vitrum heparin of Upjohn heparin penetrate with great difficulty (perhaps due to a higher polymerization). Thus, it would seem plausible that the heating of solutions of Vitrum heparin enhances its activity by effecting a partial depolymerization. Further experiments are planned to test this possibility.

Nothing definite is known about the chemical mechanism whereby heparin has its inhibitory effect. Runnström and Kriszat (17) have noticed the antagonistic actions of Vitrum heparin and ATP on membrane formation and cleavage in the eggs of *Strongylocentrotus droebachiensis*. Also, it has been found (Harding, unpublished data) that 0.002 M ATP can completely counteract the effect of 0.01 per cent to 0.025 per cent H.W.D. heparin upon fertilization. Eggs fertilized in the presence of 0.05 per cent H.W.D. and 0.002 M ATP show partially elevated membranes. This suggests that the heparin might interfere with phosphate metabolism at the egg surface. Indeed, Lindberg (8) has presented convincing evidence that ATP is metabolized at the surface of the sea urchin egg, outside the semipermeable barrier.

The opposite actions of heparin and ATP have been noted in other biological systems. Goldacre and Lorch (4) found by means of microinjection experiments with *Amoeba discoides* that "ATP causes the motion of the cytoplasm away from the site of injection, and heparin causes motion towards it." Fischer and Schmitz (2) found that ATP counteracts the effect of heparin in blood clotting. Also, McIntyre and Braverman (11) found that heparin inhibits the contraction of acto-myosin threads which normally occurs when ATP is added. This may have some relation to the observation that heparin inhibits the contraction of the egg cortex which normally occurs at fertilization (18).

Runnström has suggested that there is present in the mature, unfertilized egg a polysaccharide serving as a natural inhibitor to further development. Upon fertilization the activity of this inhibitor would have to be removed. Runnström and Kriszat (16) have found that direct treatment of the eggs *Psammechinus miliaris* with dilute solutions of sodium periodate results in a state of hypersensitivity to the activating influence of hypertonic sea water. Treatment with periodate alone was sufficient to activate about 10 per cent of the eggs. This suggests that a natural inhibitor to activation and cleavage may indeed be present in the unfertilized egg. Destruction of this inhibitor by periodate oxidation results in activation or a state of hypersensitivity to other activating agents. In this regard, it is of interest that those substances which favor normal fertilization or activation by direct treatment of the eggs, are known to be antagonistic to heparin or jelly coat substance. For example, if the eggs of *Arbacia lixula* are aged in sea water for several hours, they gradually lose their capacity to elevate normal fertilization membranes. After fertilization a thin gelatinous rim appears on the surface of the egg. Wicklund (14, p. 251) has found that treatment of *Arbacia lixula* eggs with ATP or crystalline serum albumin maintains their capacity for normal fertilization. It was also found that freshly obtained eggs in many cases are refractive to fertilization. In this case also, normal fertilization could be obtained by treatment of the eggs with ATP or serum album (14, 17).

Monné and Slautterback have obtained cytochemical evidence for the presence of polysaccharides in the egg cortex (12). These observations were made on the fertilized eggs of *Paracentrotus lividus*. Kelly (7) has observed a metachromatic staining of the cytoplasm in the living eggs of *Chaetopterus pergamentaceus,* indicating the presence of heparin-like sulfate-containing mucopolysaccharides; and Heilbrunn and Wilson (5) have emphasized the importance of such naturally occurring polysaccharides in cell division. It would be of interest to see if there is any change in the distribution or staining capacity of these polysaccharides at the time of fertilization.

Lundblad and Monroy (9) have described a mucopolysaccharase activity present in sea urchin sperm. Recently Vasseur (19) has found that the sperm of *Paracentrotus lividus* contain upon their surfaces an enzyme capable of depolymerizing the jelly coat substance. It is conceivable, as a continuation of the above line of thought, that this enzyme or enzyme complex has a function, not only upon the jelly coat substance, but also upon a polysaccharide inhibitor present at or near the egg surface. Heparin might prevent the action of this enzyme through a competitive inhibition, such as occurs in the inhibition of hyaluronidase by heparin (1, 10) thus prevent-

ing normal fertilization. However, we have already seen that heparin can inhibit membrane elevation in artificially activated eggs; and the above idea is not valid as an explanation of the heparin effect unless we assume that the polysaccharide depolymerizing enzyme is also present in the egg.

SUMMARY

1. Several polysaccharides were tested for their action on fertilization in the eggs of *Arbacia punctulata* and *Echinarachnius parma*.

2. One sample of heparin (from Hynson, Westcott, and Dunning Co.); a mucopolysaccharide, the human H substance, extracted from pig mucin; and "fucoidin," a polysaccharide preparation from *Fucus vesiculosis*, inhibited elevation of the fertilization membrane in relatively low concentrations. Heparin from the Upjohn Co. and also from the Vitrum Co. were relatively inactive. Heating the solutions of Upjohn and Vitrum heparin increased their activity. Oxidation of these polysaccharides with appropriate concentrations of sodium periodate removed their inhibiting activity.

3. Using Hynson, Westcott, and Dunning heparin and *Arbacia punctulata* eggs the following information was obtained: a) 0.2 per cent heparin prevents cleavage if the eggs are fertilized in the heparin solution. b) If the eggs are fertilized in sea water and then placed in 0.2 percent heparin three to five minutes after fertilization, cleavage occurs. This is true also if the fertilization membrane and hyaline layer are removed to allow penetration of the heparin to the fertilized egg surface. c) Heparin inhibits membrane elevation in eggs from which the jelly coats have been removed. d) Heparin inhibits the membrane elevation which normally occurs when *Arbacia punctulata* eggs are activated with hypertonic sea water.

These results are interpreted as an indication that heparin can prevent cleavage by inhibiting normal fertilization. Also, the heparin has, at least in part, an action on the protoplasmic surface of the egg.

4. Possible chemical mechanisms which might explain the inhibiting activity of heparin are discussed.

I am very much indebted to Professor John Runnström for suggesting this problem and offering much of his time for valuable discussions and criticisms. I should also like to express my indebtedness to Astri Runnström for a great deal of careful technical assistance.

REFERENCES

1. Astrup, T., and Alkjaersig, N., *Nature*, **166**, 568 (1950).
2. Fischer, A., and Schmitz, A., *Biochem. Z.*, **259**, 61 (1933).
3. Gagnon, A., personal communication.
4. Goldacre, R. J., and Lorch, I. J., *Nature*, **166**, 497 (1950).
5. Heilbrunn, L. V., and Wilson, W. L., *Proc. Soc. Exptl. Biol. Med.*, **70**, 179 (1949).
6. Herbst, C., *Arch. Entwicklungsmech.*, **9**, 424 (1900).
7. Kelly, J., *Protoplasma* (in press).
8. Lindberg, O., *Exptl. Cell Res.*, **1**, 105 (1950).
9. Lundblad G., and Monroy, A., *Arkiv Kemi*, **2**, 343 (1950).
10. McClean, D., *J. Path. Bacteriol.*, **54**, 284 (1942).
11. McIntyre, A. R., and Braverman, I., *Federation Proc.*, **6**, 158 (1947).
12. Monné, L., and Slautterback, D. B., *Exptl. Cell Res.*, **1**, 477 (1950).
13. Runnström, J., *Arkiv Zool.*, **40 A**, nr. 17 (1948).
14. —— *Advances in Enzymol.*, **9**, 241 (1949).
15. —— *Exp. Cell Res.*, **1**, 304 (1950).
16. Runnström, J., and Kriszat, G., *Exptl. Cell Res.*, **1**, 355 (1950).
17. —— *Exptl. Cell Res.*, **1**, 497 (1950).
18. Runnström, J., and Wicklund, E., *Arkiv Zool.*, **1**, No. 13 (1950).
19. Vasseur, E., (in press).

CYTOLOGICAL EFFECTS OF COLCHICINE ON THE GRASSHOPPER NEUROBLAST IN VITRO WITH SPECIAL REFERENCE TO THE ORIGIN OF THE SPINDLE[1]

MARY ESTHER GAULDEN and J. GORDON CARLSON

Biology Division, Oak Ridge National Laboratory, Oak Ridge[2], *and Department of Zoology and Entomology, The University of Tennessee, Knoxville*[3]

Received December 5, 1950

The cytological studies of Ludford (26), Nebel (28), Eigsti (15) and Levan (25) demonstrated that colchicine stops mitotic progress in the living cell by deleteriously affecting the spindle structure. Numerous studies have subsequently substantiated this conclusion and added much to the information on the cytological effects of various concentrations of colchicine on a wide variety of tissues. Since practically all these results have been based on studies of fixed and stained material, it seemed desirable that observations be made of the action of colchicine on the living cell. Studies were therefore made of hanging-drop preparations of living neuroblast cells of the grasshopper embryo. An additional incentive to the investigation was the discovery in our laboratory of the destructive action of certain wave lengths of ultraviolet radiation on the mitotic spindle; it was desired to compare in detail the action of a physical agent, such as ultraviolet radiation, with a chemical agent, such as colchicine.

MATERIAL AND METHODS

Embryos of *Chortophaga viridifasciata* (De Geer) at an age equivalent to 14 days development at 26° C are removed from the egg in artificial culture medium[4] and separated from the surrounding yolk. The amniotic membrane is split along the mid-ventral line and the maxillary and thoracic appendages cut off close to the body with fine knives. To reduce the size of the tissue mass, the head and posterior half of the abdomen are usually removed. The remaining portion of the embryo is

[1] This investigation was begun at the University of Alabama with aid of a grant from the University Research Committee and continued at the Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland.

[2] Report prepared under Contract W-7405-eng-26 for the Atomic Energy Commission at Oak Ridge.

[3] Contribution No. 41 from the Department of Zoology and Entomology.

[4] NaCl 0.68 gm, KCl 0.02 gm, $CaCl_2$ 0.02 gm, $MgCl_2$ 0.01 gm, NaH_2PO_4 0.02 gm, $NaHCO_3$ 0.012 gm, glucose 0.77 gm, water (pyrex redistilled) 100.0 cc.

mounted, ventral side against the cover glass, in a thin film of the medium containing colchicine, and the cover glass is sealed over a depression slide with heavy mineral oil.

All operations are performed aseptically, ultraviolet light serving as the sterilizing agent for instruments, slides, cover glasses, and culture medium (12). Ultraviolet irradiation alters the colchicine molecule in some way that renders it considerably less effective as a mitotic poison; therefore, it is necessary to add the desired amount of colchicine to sterilized culture medium. No contamination of the cultures has been encountered with this procedure.

The hanging-drop preparations are studied under the oil immersion lens of a microscope enclosed in a box which is maintained at 38° C. Situation of these cells just beneath the cover glass permits observations to be made continually or at desired intervals on the mitotic progress of selected neuroblasts and on the structure of their cellular components.

All observations herein reported were made on living cells that were being continuously subjected to colchicine. In addition, different preparations were fixed and stained with acetocarmine, pyronin-methyl green and iron hematoxylin, so that certain features of colchicine action difficult to determine in the living cell could be confirmed. All photographs were made from these fixed and stained preparations.

OBSERVATIONS

The lowest concentration tested, namely, 0.05×10^{-6} M or one part colchicine to 50 000 000 parts of culture medium, failed to produce any detectable abnormalities in the morphology or behavior of cells that were treated for as long as 24 hours.

The highest concentration used was 1000×10^{-6} M or one part colchicine to 2 500 parts of culture medium. For 30–60 minutes after beginning of exposure the effects resembled those produced by somewhat lower concentrations of colchicine, e.g., 50×10^{-6} M. At the end of this period, however, the cells exhibited a shrunken appearance followed by a condition resembling pycnosis which made further analysis of the mitotic and structural changes impossible. The late prophase nuclei observed during the first 30 minutes of treatment with this concentration were strikingly similar to the "ball" metaphases described by Barber and Callan (1) in colchicine treated Triturus cells.

Concentrations of 0.1×10^{-6} to 50×10^{-6} M interfered with mitosis primarily by deleteriously affecting the spindle of the prometaphase and metaphase stages. Some of these concentrations also produced effects on other stages of mitosis, i.e., late prophase and anaphase, but such effects are less frequently observed than are those involving the spindle and are of less significance in blocking mitotic progress.

The presentation of the results obtained with concentrations of 0.1×10^{-6} to 50×10^{-6} M is made difficult because five variables are involved: (1) the concentration of colchicine used, (3) the duration of exposure of the cell to colchicine, (2) the mitotic stage of the cell at the time treatment began, (4) the mitotic stage of the cell at which effects appear, and (5) the structures of the cell which are affected as well as the extent of the change produced in the form and behavior of these structures. In view of these variables, the results will be discussed first from the standpoint of the immediate effects of exposure to high concentrations, and second, from the standpoint of the effects of prolonged exposure to low concentrations of colchicine.

1. IMMEDIATE EFFECTS OF EXPOSURE TO HIGH CONCENTRATIONS

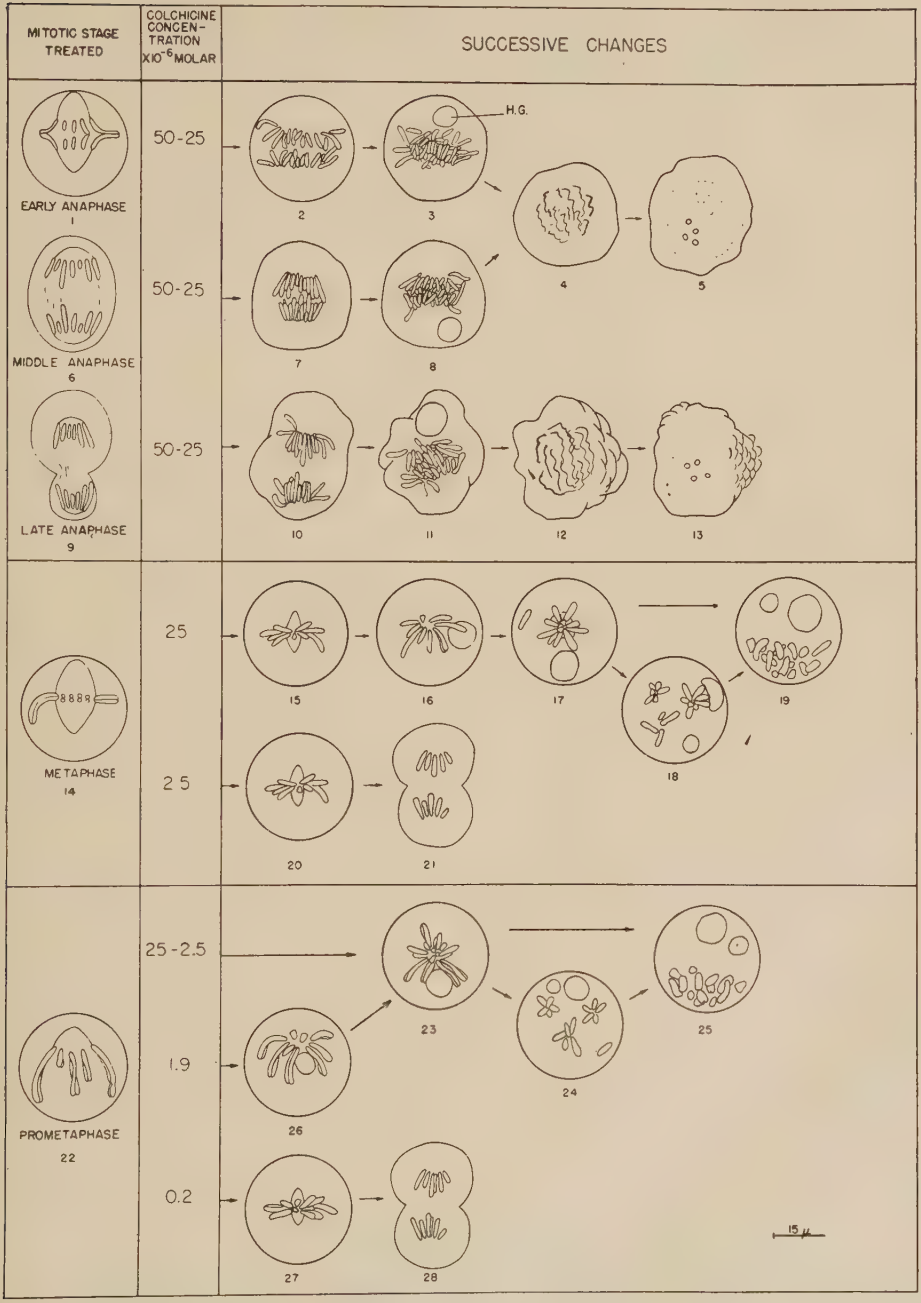
a. *Anaphase*

50 and 25 $\times$ 10^{-6} M. The spindle is impaired almost immediately with the result that separation of the daughter chromosomes[1] ceases. Gradually the partially separated daughter chromosomes move toward each other and intermingle (figs. 1–13, 43, 44). A hyaline globule (11) is usually formed close to the chromosome mass. In all cells in which the spindle structure is destroyed or in which spindle formation is prevented, the spindle material takes one of two forms: 1) it usually goes into the formation of one or several spherical bodies which we have called "hyaline globules," or 2) less frequently it becomes dispersed with the disoriented chromosomes and thus has no definite shape. These observations and their significance are discussed later (p. 9).

The telophasic transformation of the chromosomes is delayed only slightly as compared with that in normal cells. Four nucleoli, which never attain normal size and shape, are formed in the tetraploid nucleus (figs. 5, 13) in contrast to the two found in the normal diploid nucleus of the grasshopper neuroblast. Cleavage is completely suppressed. Cells treated in early or middle anaphase become irregular in outline while cells treated in late anaphase, in which a cleavage furrow is evident, develop pseudopod-like processes which are rapidly extended and withdrawn from the free surface of the cell for several hours. Eventually the entire periphery of such cells consists of small blebs (fig. 13). Bleb formation after colchicine treatment has also been observed by Ludford (26) and Miszurki and Doljanski (27), in fibroblasts. Mustard gas (20) and ultraviolet radiation (11) can induce pseudopod and bleb formation. It appears, therefore, that colchicine, as well as other experimental agents, can augment and intensify the changes normally occurring at the cell surface during cleavage.

Nuclear membrane formation does not take place in these tetraploid neuroblasts, and the boundary between nucleus and cytosome is not very sharp. It must be kept in mind, however, that these cells were bathed by a strong concentration of colchicine

[1] The twenty-three chromosomes per cell in the male and the twenty-four in the female are all rod-shaped with terminal centromeres.



Figs. 1–42 are adapted from camera lucida drawings.
Figs. 1–28. Effects of colchicine on the mitotic spindle. H. G., hyaline globule.

throughout the observation period (4 hours), and this may be instrumental in preventing the formation of a definite nuclear membrane. On the other hand the absence of spindle material or karyolymph precursor about the chromosomes (the spindle material formed a hyaline globule) may account for the absence of nuclear membrane formation.

If the cells treated in anaphase had been removed from the colchicine solution, a nuclear membrane might have formed, and, if so, this could be one possible method whereby tetraploid cells arise. Rosenfeld (32), using ether on fibroblasts, and Levan (25), using colchicine on onion root tips, have also reported that tetraploid cells can be produced by treating cells in anaphase.

2.5×10^{-6} M. No detectable effects are produced on anaphase by this concentration.

b. *Metaphase*

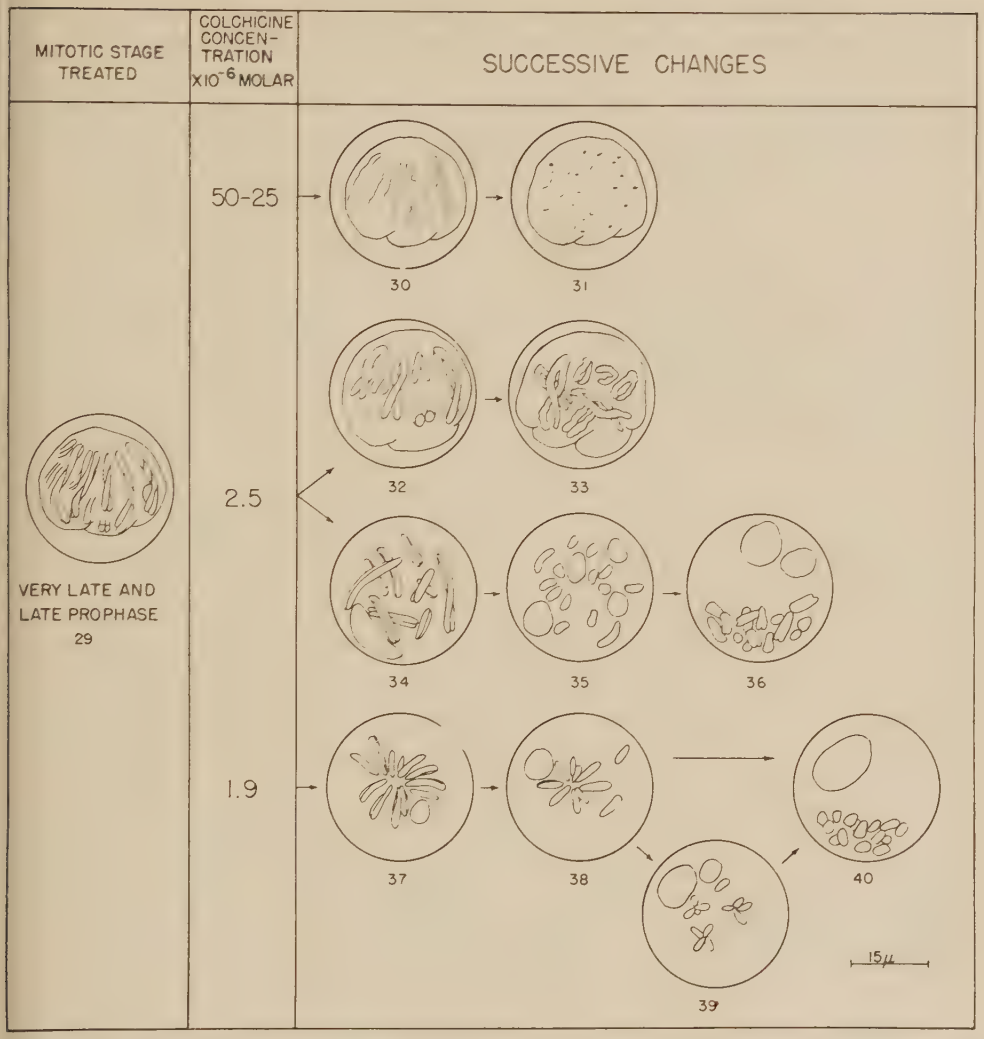
25×10^{-6} M. The fully formed metaphase spindle gradually decreases in size, drawing the proximal ends of the chromosomes toward a common center until they are almost contiguous. Simultaneously the distal ends of the chromosomes move off the equatorial plane so that they finally radiate outward in all directions (figs. 14–17, 45–48) to form a "star" metaphase (1). The spindle material is at the same time forming a hyaline globule. Sometimes one or several chromosomes will escape from the focal point (figs. 17, 49). The star metaphase persists for several hours. Often the small amount of spindle material holding the chromosomes together divides into two or more parts, each serving as a focal point of attraction for a few chromosomes (figs. 18, 50). Later the focal point or points disappear and the chromosomes lose all orientation. After complete destruction of the spindle the cytoplasm undergoes a lowering of viscosity, as evidenced by the increased rapidity of Brownian movement of the mitochondria, and the chromosomes settle to the lower half of the cell. During this whole course of events, which occupies about 5–6 hours, the chromosomes have become shorter and thicker. They are individually distinguishable as long as the cell is in the colchicine solution (fig. 19).

2.5 and 2.0×10^{-6} M. This lower concentration reduces the size of the spindle, but the reduction is insufficient to prevent division. The abnormally small spindle locates in the middle of the cell (fig. 20), the cleavage furrow forms at the equator (fig. 21), and the daughter cells produced are nearly equal in size; thus they differ from the untreated cell in which the spindle is not centered with the cell and from which two cells of unequal size are produced. The metaphase-anaphase stages are of longer duration at this concentration, i.e., 30–40 minutes in contrast to an average of 22 minutes in untreated neuroblasts (11). Hence mitosis is retarded but not blocked.

1.9×10^{-6} M. No visible effect is produced on cells treated in metaphase with this concentration.

c. *Prometaphase*

25 to 2.5×10^{-6} M. The amount of organized spindle material holding the chromosomes together at prometaphase is immediately reduced in size (fig. 23). The result-



Figs. 29-40. Effects of colchicine on cells treated in late prophase and very late prophase. The shape of the cells illustrated is that of very late prophase; late prophase cells are concavo-convex in shape.

ing focal point soon disappears and the chromosomes scatter as described above for cells treated in metaphase with 25×10^{-6} M solution.

1.9×10^{-6} M. A very small spindle is formed (figs. 26, 53), but it is soon reduced to a focal point which often breaks up into several focal points. Finally, about 5–6 hours after beginning of treatment, all orientation is lost, and the chromosomes settle to the lower half of the cell.

0.2 × 10⁻⁶ M. A small spindle forms in the center of the cell. Division is delayed about 30 minutes and results in two cells of equal size. The same effect, it will be noted, occurs when cells are treated at metaphase by a stronger concentration, namely, 2.5×10^{-6} M.

0.1 × 10⁻⁶ M. No visible effects are produced on cells treated at prometaphase by this or lower concentrations of colchicine.

d. *Middle and Late Prophase*

50 and 25 × 10⁻⁶ M. The nuclei revert to early prophase, i.e., the chromatin assumes an appearance similar to the chromatin in early prophase nuclei (figs. 30, 31). Such reversion occurs within 10–20 minutes after the beginning of treatment. After much delay the cell progresses to prometaphase where it is blocked by destruction of the spindle structure. The total time from initiation of treatment to prometaphase is about 150 minutes. (The combined duration of middle and late prophase in the untreated neuroblast is about 32 minutes.)

2.5 × 10⁻⁶ M. The majority of cells treated at middle or late prophase with this concentration progress to prometaphase with no delay. When the nuclear membrane disappears, however, no spindle is formed, and consequently the chromosomes are unoriented in the cell (figs. 34, 54).

Occasionally cells treated in late prophase with 2.5×10^{-6} M concentration will be retarded, i.e., breakdown of the nuclear membrane will be delayed. The chromosomes of such cells shorten and their chromatids become somewhat separated from each other except for a few relational coils, so that the chromosomes have a diakinesis-like appearance (fig. 32). Similar cells are found in rare instances in untreated grasshopper embryos, but the incidence of retarded late prophases is considerably greater in colchicine-treated cells than in untreated ones.

Cells that have been blocked at late prophase for several hours occasionally exhibit a further effect of colchicine, namely, the chromosomes lose their orientation in the nucleus so that the centromere ends are no longer arranged in a circle about the nuclear core at the polar side of the nucleus (fig. 33) but lie at random therein. This finding, which may have a bearing on the interpretation of the organization of prophase nuclei and origin of the spindle, is discussed later (p. 10).

1.9 × 10⁻⁶ M. Cells treated in middle or late prophase with this concentration pass to prometaphase unaffected. The behavior of the cell after nuclear membrane breakdown is similar to that described above for prometaphases subjected to this concentration.

1.65 × 10⁻⁶ M. No visible effects are produced by this and lower concentrations.

e. *Telophase, Interphase and Early Prophase*

None of the concentrations used in this study produces any immediate effect on the structure and behavior of these stages. Treatment with $1\,000 \times 10^{-6}$ M results in pycnosis, but, as pointed out earlier, all cells, regardless of mitotic stage, suffer disintegration when subjected to this concentration of colchicine.

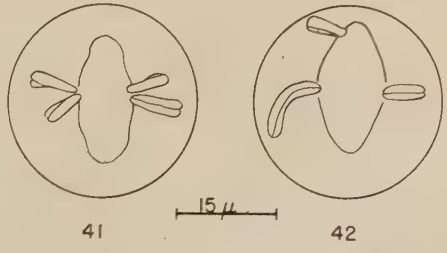
2. EFFECTS OF PROLONGED EXPOSURE TO LOW CONCENTRATIONS

0.2 × 10⁻⁶ M. There are no immediately detectable effects of this concentration of colchicine on mitosis. Cells which enter or are already in metaphase when first subjected to the solution progress through anaphase to telophase unaltered. The first disturbance to be observed usually occurs about 1 hour after the beginning of treatment. At this time cells in mid-anaphase frequently exhibit a cleavage furrow which is situated at the equator rather than at the lower third of the cell. When the cell reaches late anaphase the furrow usually shifts to the lower third of the cell, thus resulting in a normal unequal division. Ninety minutes after the beginning of treatment, however, practically all cells are dividing equally and therefore abnormally.

After exposure to this concentration of colchicine for 2 hours, all cells entering metaphase develop very small spindles which are gradually reduced in size and completely destroyed 5–6 hours later. The effectiveness of colchicine is so diminished at this concentration that a small amount of spindle material, constituting a focal point, can still be organized at nuclear membrane breakdown 6–7 hours after beginning of treatment, but it is soon destroyed.

0.1 × 10⁻⁶ M. There are only two minor manifestations of colchicine action during the first 6 hours of treatment with this concentration, and these do not appear until 2 hours after treatment begins. The first of these consists of a slight flattening of the metaphase cell at the two polar regions. The second effect observed is a slight irregularity in the outline of the spindle of some metaphases (fig. 41). These abnormalities neither alter the duration of metaphase nor disturb the subsequent cleavage. From the 6th hour of treatment through at least the 24th, a small spindle is formed at metaphase. Although cells in this stage are somewhat retarded, they eventually reach anaphase.

One phenomenon which has been seen occasionally after treatment of neuroblasts with ultraviolet radiation or with heat was observed in some of the cells treated with 0.1×10^{-6} M concentrations of colchicine. Three or four hours after the beginning of treatment several cells in metaphase exhibited one chromosome that was not oriented on the equator of the spindle but was lying at or near one pole of the spindle (fig. 42). The centromere of this chromosome was associated with the spindle and slowly moved toward the equatorial plate. Only after it had become aligned with



Figs. 41 and 42. Effects of prolonged exposure to 0.1×10^{-6} M colchicine. Fig. 41. Spindle is only slightly affected. Fig. 42. One chromosome has failed to progress to equator of spindle.

the other chromosomes (about 1 hour after the beginning of metaphase) was anaphase initiated. In one such cell fission was not completed, therefore a cell containing two nuclei was formed.

3. THE HYALINE GLOBULE

In view of the significance of the hyaline globule with reference to its possible bearing on the problem of the mode of action of spindle-destroying agents as well as on the question of the origin of the spindle, the formation and properties of this body will be discussed in some detail.

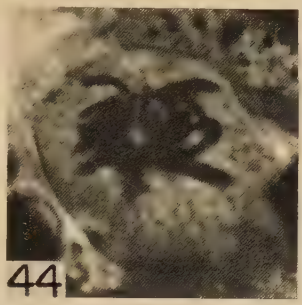
Spindle formation in untreated neuroblast. Before discussing the formation of the hyaline globule it might be well to review the salient features of spindle formation in the untreated neuroblast. The centromere ends of the chromosomes at prophase are arranged in a circle about the nuclear core at the polar side of the nucleus (fig. 29) and their distal ends extend into the apolar portion of the nucleus. An opaque, optically homogeneous karyolymph of apparently much higher viscosity than the cytoplasm fills the nucleus. This high viscosity is evidenced by the failure of the mitochondria, which are abundant in the cytoplasm, to penetrate the karyolymph after disappearance of the nuclear membrane. In fact, the karyolymph, and subsequently the spindle, are recognized as such solely by the almost complete absence of mitochondria. The spindle in the living neuroblast, as observed with bright-field illumination, reveals none of the fibrous structure often seen in fixed and stained preparations.

The spindle first makes its appearance about 30 seconds after nuclear membrane breakdown; one pole becomes visible in the region around the proximal ends of the chromosomes and is indistinguishable optically from the karyolymph with which it is continuous. The spindle gradually increases in size during prometaphase, apparently using the karyolymph in its formation. During the growth of the spindle the terminal centromeres move outward and away from the polar side of the cell to occupy at metaphase a position on the edge of the spindle equator. The distal portions of the chromosomes come to lie in the cytoplasm and are surrounded by a thin layer of parachromosomic material which is continuous proximally with the spindle.

Figs. 43–54 are photographs taken from sections (13 μ thick) of grasshopper embryos. Unless otherwise stated the cells were stained with iron hematoxylin.

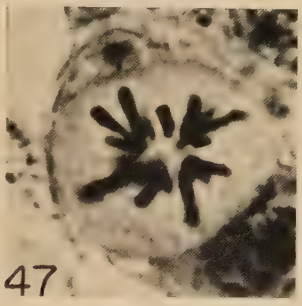
43. Elongation of anaphase spindle has stopped and chromosomes are losing orientation. 50×10^{-6} M, 10 minutes.
44. Daughter chromosomes of anaphase are intermingling. 50×10^{-6} M, 10 minutes.
45. Untreated metaphase. Cell stained with pyronin-methyl green.
46. Reduced spindle. 25×10^{-6} M, 30 minutes.
47. Star metaphase. 2.5×10^{-6} M, 90 minutes.
48. The clear spheres around the star are hyaline globules. 2.5×10^{-6} M, 90 minutes.
49. Four chromosomes have become separated from a star. 2.5×10^{-6} M, 120 minutes.
50. Three stars in one cell. 2.5×10^{-6} M, 180 minutes.
51. Disoriented contracted chromosomes. 2.5×10^{-6} M, 180 minutes.
52. Position of chromosomes as determined by gravity. 2.5×10^{-6} M, 300 minutes.
53. Reduced prometaphase spindle. 1.9×10^{-6} M, 30 minutes.
54. Disoriented prometaphase chromosomes. Cell was in colchicine solution when nuclear membrane disappeared. 2.5×10^{-6} M, 15 minutes.

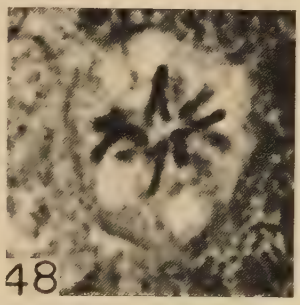
43


44

45

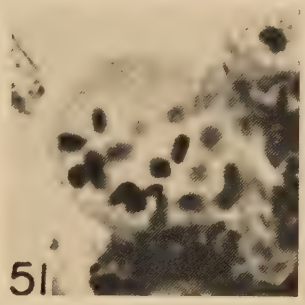
46

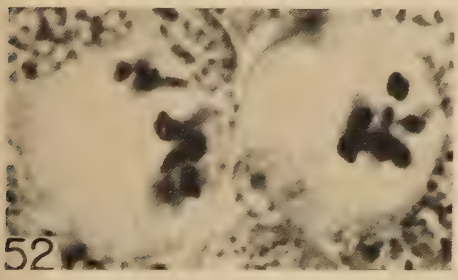

47


48

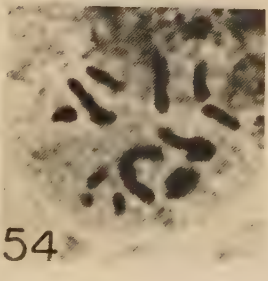
49

50


51


52

53


54

15μ

Globule formation in the treated neuroblast. The hyaline globule can originate either from the karyolymph of late prophase or from the spindle of metaphase or anaphase when the cell is subjected to an effective concentration of colchicine. In the former instance the globule begins to form within a few minutes after the breakdown of nuclear membrane. The chromosomes lose their late prophase orientation. At the same time the karyolymph starts flowing to one side, so that at the end of 20–60 minutes a large part of it is incorporated in one or several of these globules (fig. 34). The disoriented chromosomes often appear to be connected with each other and the globule material by a remaining small amount of karyolymph which never goes into the formation of the globule. Often when there are several globules present on the same side of the cell, small "canals" can be seen connecting them. Once formed, a globule persists as long as colchicine is present.

Formation of a globule from a metaphase spindle requires a longer period of time than does formation directly from the karyolymph, the exact duration depending on the concentration of colchicine used and on the degree of formation of the spindle when first exposed to the colchicine. The first evidence of the colchicine effect is a gradual reduction in the size of the spindle. When the spindle has been reduced to about half its initial size, a small hyaline globule appears at one side of the chromosome group; it increases in size with further reduction of the spindle to a focal point, and is finally completely destroyed (figs. 14–19).

Globule formation is also observed after spindle destruction in anaphase (25 and 50×10^{-6} M colchicine). Usually only one globule appears, and it is smaller than those found in cells treated at prometaphase or metaphase. In most cases this globule lies in that region of the cell formerly occupied by one pole of the spindle. Whether it forms at one or the other of the poles seems to be a matter of chance. Actual formation of the globule in anaphase remains obscure because the spindle was not observed flowing into the globule—the globule just appeared soon after colchicine began to exert its influence.

Characteristics of the hyaline globule. The spindle substance making up the hyaline globule is typically spherical in form, unless pressure between the chromosome group or other globules and the cell membrane flatten it somewhat (fig. 34). The slight irregularity in its shape makes precise measurements difficult, but the diameter varies from about 3 to 15 μ, depending on the age and the number of globules in a cell. The initial formation is so rapid that the globule is several microns in diameter before it is first observed. If only one is formed in a cell, it may attain a diameter of approximately 15 μ which, it will be noted, is over half the average diameter of the cell itself. The time required to reach this size depends on the speed with which the spindle material is affected by a given concentration of colchicine. If several globules are formed in a cell, they will be smaller than a solitary one and of unequal size, e.g., there may be one 8, one 10, and one 12 μ in diameter. The larger single globules which are formed initially will sometimes break up into five to eight smaller ones after the cell has been subjected continuously to colchicine for 6 hours or more. In rare instances the spindle material, instead of forming a spherical body, will assume a half-moon shape and even more rarely it will become oblong shaped. In such cases the structure will not exceed 18–25 μ in length, and it will be somewhat flattened toward the polar and apolar regions of the cell.

In the living neuroblast the material of the hyaline globule is optically indistinguish-

able from that of the karyolymph and the spindle. It is an opaque, homogeneous substance having a high viscosity. It is not surrounded by a membrane.

The position of the hyaline globule in the cell shifts with the duration of exposure to colchicine, and therefore with the decreasing viscosity of the cytoplasm. The hyaline globule, when first formed, lies to one side of the chromosome group, its position with reference to the cell's polarity being a random one. Later, however, the position of the cellular components seems to be determined by gravity; the chromosomes tend to move downward and the globule moves upward. Several hours after spindle destruction, the globule is invariably found at the top of the cell and all the chromosomes in the lower half of the cell (fig. 19). The viscosity of the cytoplasm has by this time become extremely low, which probably accounts for this distribution of chromosomes and globules. By centrifuging mitotic cells of wheat, Beams and King (3) have shown that the spindle substance is lighter and the chromosomes heavier than the cytoplasm. Thus, in colchicine-treated neuroblasts the rise of the hyaline globule and attendant fall of the chromosomes lends further support to the contention that the hyaline globule is derived from the spindle material.

4. ADDITIONAL OBSERVATIONS

No evidence was found to indicate that colchicine produces chromosome breakage, alters mitochondria size, or accelerates any stage of the mitotic cycle of the grasshopper neuroblast.

DISCUSSION

Spindle. The primary, and indeed the most commonly observed, effect of colchicine in the cell is its destructive action on the mitotic spindle. Figures 1–28 show schematically how the reaction of the spindle to colchicine varies with the stage of mitosis initially treated and with the concentration of the alkaloid. Three main points stand out. (1) The greater the concentration of colchicine, the greater will be its effectiveness in destroying the spindle or interfering with its development. (2) The more completely the spindle is developed at the time of exposure to colchicine, the greater is the concentration required to destroy it or prevent its further development. Hence the spindle material is most easily affected at very early prometaphase (2). The series of changes in chromosome orientation that take place during colchicine action are directly related to successive stages in the destruction of the spindle. At a very low concentration (0.1×10^{-6} M) the spindle is only slightly reduced (fig. 41), but as the strength of colchicine increases, a proportionately greater reduction of the spindle body takes place (figs. 15–19).

One of the more frequently observed configurations in colchicine-treated neuroblasts is the "star" metaphase (figs. 17, 23). Star formation after colchicine treatment was first illustrated by Pernice in 1889 (18) in cells

of dog intestine. Ludford (26) and Levan (25) noted stars in colchicine-treated cells and interpreted them as evidence of a multipolar spindle. Several investigators, notably Barber and Callan (1) and Peters (31), attribute the occurrence of stars to monaster formation brought about by a disturbance in the behavior of centromeres, centrosomes, and chromosomal fibers. Observations on living neuroblasts reveal that stars can be formed in two ways. First, they may appear during either partial or complete destruction of a fully formed spindle body by strong concentration of colchicine. Second, if the concentration of colchicine within the cell is of medium strength, enough spindle material will be organized at prometaphase to form the focal point of a star. Thus, we see why Barber and Callan (1) observed stars most frequently during the first hours of exposure to a relatively strong solution, and why Levan (25) and Peters (31) observed them most frequently during recovery when the intracellular concentration of the alkaloid was being diluted. Since the poles of the spindle can be seen approaching the chromosomes during spindle diminution in neuroblasts (fig. 15), it appears not unlikely that the vestige of the spindle structure which holds the chromosomes together in a star is composed of polar material (centrioles are not visible in grasshopper cells).

Hyaline globule. Even though the literature abounds in studies of the action of colchicine on mitosis in both plants and animals (16, 17), there are relatively few reported observations of stuctures which resemble the hyaline globule herein described. "Vacuoles" amidst scattered chromosomes were seen in colchicine-treated fibroblasts by Bucher (6). Shimamura (33) and Berger and Witkus (4) also noted spherical, unstained bodies in colchicine-treated onion cells and suggested that they represented material from the disorganized spindle. The "distorted star" metaphase of colchicine-treated newt cells described by Barber and Callan (1), probably represents formation of a hyaline globule which is displacing the radial arrangement of the chromosomes. Several investigators (26, 30, 7) have called attention to a structure which resembles the hyaline globule except that it forms a halo around the chromosomes. According to Paletta and Cowdry (30) the halos do not stain, they do not contain mitochondria, they vary in size, and they retain their shape after ultracentrifugation. These characteristics resemble those of the hyaline globule. All the above reports deal with fixed cells. Living *Arbacia* eggs treated with colchicine have been examined (2), and the disappearance of the astral rays and the subsequent appearance of "lake-like" bodies have been observed as the alkaloid penetrated the eggs.

It seems very likely that all these authors are describing a structure which

is similar to the hyaline globule of the colchicine-treated grasshopper neuroblast not only in appearance but in origin. Since the hyaline globule is often rather difficult to preserve in fixed and stained preparations of neuroblasts, the paucity of reports of its occurrence may be explained, in part at least, by the fact that most of the work that has been done with colchicine has made use of fixed materials only.

It has been suggested by Bernal (5) and others that the spindle is a tactoid or liquid crystal, and that its existence as a structure, therefore, is dependent upon the fibrous shape of its molecules. With this as a basis of explanation for the normal spindle structure, Östergren (29) has postulated that the ability of c-mitotic substances to destroy the spindle substance lies in their affinity for its protein molecules. The substances become associated with the fibrous spindle molecules causing them to fold into a more or less spherical shape, thereby bringing about a destruction of the tactoid-like spindle structure.

In this connection, it is of considerable interest that, under the influence of colchicine, the spindle substance of the grasshopper neuroblast forms, in most cases, a spherical body. Furthermore, this body, the hyaline globule, in fixed and stained condition does not exhibit the internal fibrous structure which can be so clearly demonstrated in the fixed and stained untreated spindle. These observations lend credence to the hypothesis that colchicine acts not by destroying the spindle material but rather by altering it in some way which destroys or prevents, as the case may be, the molecular orientation necessary for the formation of a normal spindle structure.

Since in living neuroblasts the karyolymph gives rise to the spindle, which in turn, under the influence of colchicine, gives rise to the hyaline globule, it is not unreasonable to assume that the material of all three is the same in many respects and that perhaps the main difference is, in fact, one of molecular orientation. It is rather tempting to suggest that colchicine causes the spindle material to revert, so to speak, to a condition similar to, though perhaps not exactly like, that of the karyolymph, especially in view of the fact that the hyaline globule resembles the karyolymph in its lack of demonstrable fibrous structure.

Prophase chromosomes. While colchicine exerts its main effect on the spindle, it is not, contrary to general belief, without influence on the structure and behavior of the chromosomes of prophase nuclei. All effects on prophase nuclei cause mitotic delay. In the grasshopper neuroblast the action of colchicine on prophase chromosomes is manifest in three ways.

1) The stronger concentrations (50 and 25 $\times 10^{-6}$ M) cause the chromatin

of middle and late prophase nuclei to revert to a state closely resembling the chromatin of early prophase nuclei. The exact nature of this reaction of chromatin is not known, but since a diverse group of other experimental agents has been shown to induce reversion—X radiation (8), ultraviolet radiation (11), heat (21), nitrogen and carbon dioxide (24)—the alteration of a rather fundamental cellular process may be involved. Since reversion appears to be a loss of something from the chromosome which results in a decrease in its diameter, those agents which induce reversion may in some way be acting on the nucleic acid or nucleoprotein of the chromosomes.

2) An abnormal contraction of late prophase chromosomes occurs occasionally at concentrations around 2.5×10^{-6} M. Such chromosomes show their coiled nature more clearly than do chromosomes of untreated late prophase nuclei (fig. 33). These factors, together with a precocious loss of most of the relational coiling of the chromatids, give the chromosomes a diakinesis-like appearance. This phenomenon appears to be due primarily to a delay in the breakdown of the nuclear membrane, during which interval the chromosomes continue to contract. A more direct effect on the chromosomes is not to be excluded completely, however, since the contracted chromosomes in colchicine-treated late prophases show their coiled nature quite clearly. Creech (13) observed diakinesis-like chromosomes in fibroblast cultures containing hydrocarbons and attributes the phenomenon to a development of the chromosomes which is in advance of the development of the remaining structures of the cell. Peters (31) evidently encountered the same effect in colchicine-treated Triturus cells. It is of interest that Erickson and Rosen (19) found contracted chromosomes at late prophase in corn root tips treated with protoanemonin, a substance which inhibits mitosis at interphase and prophase.

3) Chromosomes of greatly retarded late prophase nuclei lose their characteristic anaphase-like orientation and become disoriented within the confines of the nuclear membrane as shown in figure 33. This observation is of interest because it suggests that the orderly arrangement of the proximal ends of the chromosomes in late prophase is maintained by some "factor" in the nucleus and that this factor can be altered by colchicine. Additional evidence for a fixed position of centromere ends of the chromosomes is to be found in the microdissection studies of Carlson (unpublished). By introducing a microneedle into late prophase nuclei of neuroblasts, he found that the proximal ends of the chromosomes are rigidly fixed at the polar side of the nucleus—very strong movement is necessary to dislodge them. The distal parts of the chromosomes, on the other hand, can be moved with

relative ease, the only restriction on their movement being the highly viscous karyolymph which surrounds them. Further, there is evidence from X-ray studies (Carlson, unpublished) that the position of the proximal ends of the chromosomes with respect to one another is disturbed very little, if any, during prophase. When an X-ray-induced translocation involves the proximal regions of two chromosomes, the subsequent metaphase configuration may show several chromosomes crowded into the short region of the spindle between the centromeres of the dicentric with a much more liberal spacing of the remaining chromosomes of the metaphase plate. This can best be explained by assuming that the arrangement of the centromeres with respect to each other remains unaltered during prophase contraction of the chromosomes as well as during the increase in spacing of the chromosomes occurring with progressive spindle development during prometaphase. Whether the orientation of the centromere ends of chromosomes in nuclei is maintained by the centromeres themselves, by the polar karyolymph, by both, or by some other device remains to be determined, but there can be little doubt that it is not fortuitous.

Cytoplasm. A change in cytoplasmic viscosity after colchicine treatment observed by Beams and King (3), Beams and Evans (2), and Wilbur (34) has also been observed in the neuroblast. Colchicine-treated neuroblasts show little or no swelling, so it seems unlikely that the decrease in cytoplasmic viscosity of these cells is due to an increase in the water content of the cells. Carlson (9) has suggested that the amount of ribonucleic acid in the cytoplasm may determine its viscosity. Since Davidson, Leslie, and Waymouth (14) have found that colchicine reduces the ribonucleic acid phosphorus content of cells, the extremely low viscosity prevailing in c-mitotic neuroblasts may be due to an abnormally low level of ribonucleic acid in the cytoplasm.

SUMMARY

The effectiveness of colchicine in destroying or preventing the development of the spindle is determined by the concentration of the colchicine and the degree of development of the spindle at the time of exposure; the greater the concentration of colchicine, the greater will be its effectiveness in destroying the spindle or interfering with its development; the more completely the spindle is developed at the time of exposure to colchicine, the greater is the concentration of colchicine required to destroy it or prevent its further development.

The series of changes in chromosome orientation that take place during

the course of colchicine action, namely, approximation of centromeres, formation of "stars," the breaking up of one "star" into several, and complete chromosome disorientation, represent successive stages in the destruction of the spindle.

Destruction of the completely formed spindle is typically accompanied by the accumulation of the spindle material outside the diminishing spindle in a hyaline globule. Similarly, interference of colchicine with spindle development leads to the accumulation of the presumptive spindle material, i.e., the karyolymph, in one or more hyaline globules. It is suggested that colchicine does not destroy the spindle material but merely alters its molecular orientation, so that it comes to comprise a spherical mass with no mitotic function.

Strong concentrations of colchicine cause middle and late prophase nuclei to revert to early prophase. Somewhat lower concentrations applied to late prophase nuclei occasionally delay the breakdown of the nuclear membrane without altering the rate of chromosome contraction. In greatly retarded late prophase nuclei the chromosomes lose their intranuclear orientation, which lends support to the concept that the centromeres normally maintain a fixed position within the nucleus.

REFERENCES

1. Barber, H. N., and Callan, H. G., *Proc. Roy. Soc.*, **131**, 258 (1943).
2. Beams, H. W., and Evans, T. C., *Biol. Bull.*, **79**, 188 (1940).
3. Beams, H. W., and King, R. L., *Biol. Bull.*, **75**, 189 (1938).
4. Berger, C. A., and Witkus, E. R., *Bull. Torrey Bot. Club*, **70**, 457 (1943).
5. Bernal, J. D., The Cell and Protoplasm, Science Press, 1940.
6. Bucher, Otto, *Z. Zellforsch. u. Mikroskop. Anat.*, **29**, 283 (1939).
7. Buschke, W., Friedenwald, J. S., and Fleischmann, W., *Bull. Johns Hopkins Hosp.*, **73**, 143 (1943).
8. Carlson, J. G., *J. Morphol.*, **66**, 11 (1940).
9. —— *Biol. Bull.*, **90**, 109 (1946).
10. —— *Anat. Rec.*, **105**, 499 (1949).
11. Carlson, J. G., and Hollaender, A., *J. Cellular Comp. Physiol.*, **31**, 149 (1948).
12. Carlson, J. G., Hollaender, A., and Gaulden, M. E., *Science*, **105**, 187 (1947).
13. Creech, E. M. H., *Am. J. Cancer*, **35**, 191 (1939).
14. Davidson, J. N., Leslie, I., and Waymouth, C., *Biochem. J.*, **44**, 5 (1949).
15. Eigsti, O. J., *Proc. Natl. Acad. Sci.*, **24**, 56 (1938).
16. —— *Lloydia*, **10**, 65 (1947).
17. Eigsti, O. J., and Dustin, P., Jr., *Lloydia*, **12**, 185 (1949).
18. Eigsti, O. J., Dustin, P., Jr., and Gay-Winn, N., *Science*, **110**, 692 (1949).
19. Erickson, R. O., and Rosen, G. U., *Am. J. Bot.*, **36**, 317 (1949).
20. Fell, H. B., and Allsopp, C. B., *Cancer Res.*, **8**, 145 (1948).
21. Gaulden, M. E., *J. Tenn. Acad. Sci.*, **23**, 162 (1948).
22. Gaulden, M. E., and Carlson, J. G., *Genetics*, **32**, 87 (1947).
23. —— *J. Tenn. Acad. Sci.*, **24**, 104 (1949).
24. Gaulden, M. E,, Carlson, J. G., and Tipton, S. R., *Anat. Rec.*, **105**, 16 (1949).

25. Levan, A., *Hereditas*, **24**, 471 (1938).
26. Ludford, R. J., *Arch. Exptl. Zellforsch. Gewebezücht.*, **18**, 411 (1936).
27. Miszurski, B., and Doljanski, L., *Proc. Soc. Exptl. Biol. Med.*, **64**, 334 (1947).
28. Nebel, B. R., *Biol. Bull.*, **73**, 351 (1937).
29. Ostergren, G., *Hereditas*, **30**, 430 (1944).
30. Paletta, F. X., and Cowdry, E. V., *Am. J. Path.*, **18**, 291 (1942).
31. Peters, J. J., *J. Exptl. Zool.*, **103**, 35 (1946).
32. Rosenfeld, M., *Arch. Exptl. Zellforsch. Gewebezücht.*, **12**, 570 (1932).
33. Shimamura, T., *Cytologia*, **9**, 486 (1939).
34. Wilbur, K., *Proc. Soc. Exptl. Biol. Med.*, **45**, 696 (1940).

NUCLEOLAR CHANGES INDUCED IN THE GRASSHOPPER NEUROBLAST BY DIFFERENT WAVELENGTHS OF ULTRAVIOLET RADIATION AND THEIR CAPACITY FOR PHOTORECOVERY

J. GORDON CARLSON and RACHEL D. McMASTER

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee[1], *and Department of Zoology and Entomology, The University of Tennessee, Knoxville, Tennessee*[2]

Received December 19, 1950

In previous studies (2, 4) it was found that the nucleolus of the grasshopper embryo is drastically altered in form by relatively small doses of 2250 and 2537 Å ultraviolet radiation. It is changed from an indistinctly outlined, irregularly shaped body to a cluster of highly refractile spherules, which, if the dose is sufficiently large, fuse with each other subsequently to form a single, large spherical body.

The present study was undertaken to investigate in more detail the effect of ultraviolet radiation on the nucleoli, to determine within what range of wavelengths spheration is produced and the relative effectiveness of the different wavelengths in producing it, the relation of dose and wavelength to the time of appearance of this effect, the site of action of the radiation, and whether or not recovery from spheration is aided by posttreatment with visible light.

Much of the experimental work of the present study was completed at the time Kelner (10) reported a striking reactivation by visible light of conidia of *Streptomyces griseus* which had been inactivated by ultraviolet light of wavelength 2537 Å. Dulbecco (7) later demonstrated the same phenomenon with coli-bacteriophage of the "T" group, finding photoreactivation with visible light below 4400 Å. In view of the fact that our completed experiments had not been controlled with respect to exposure to visible light, it seemed advisable to test the effect of this on ultraviolet-induced spheration of the nucleolus. Actually the results obtained from the original

[1] Work performed under contract number W-7405-Eng-26 for the Atomic Energy Commission at Oak Ridge, Tennessee, U.S.A.

[2] Contribution No. 42 from the Department of Zoology and Entomology of The University of Tennessee.

experiments, in which the cells were exposed to the artificial illumination of the laboratory during and after treatment and intermittently to the light of the microscope lamp during observations, correspond closely to the results obtained when the embryos were kept in the dark or in yellow light and not exposed to intense visible light after irradiation as in the photorecovery tests. The data of the earlier studies, however, have been omitted from the present paper.

MATERIALS AND METHODS

Neuroblasts of the embryo of the grasshopper (*Chortophaga viridifasciata*) were made into hanging-drop preparations according to the method described previously (1, 3). The neuroblasts on one side of the mid-ventral line were shielded from ultraviolet radiation by a small piece of black paper attached to the top of the quartz cover directly above the embryo. Thus the treated neuroblasts of one side of the embryo could be compared readily with the untreated controls of the other side. Those neuroblasts adjacent to the mid-ventral line of the embryo were not used in determining the nucleoli affected, however, because they might have been reached by scattered radiation. Immediately after treatment the embryo was transferred from the quartz to a glass cover and placed for observation on the stage of the microscope, which was enclosed, except for the upper part of the stand and body, in a box maintained at a constant temperature of 38 $\pm$ 0.5° C.

At 10- to 15-minute intervals after treatment microscopic observations were made of the condition of the nucleoli in certain selected cells. Only nucleoli of cells close to the cover glass were studied in order to avoid, as far as possible, errors produced by absorption of the ultraviolet before it reached the nucleolus. The number of cells affected, the mitotic stages of the cells, and the time of the appearance and disappearance of spheration for each of the two nucleoli in each cell were recorded.

Certain of the preparations were treated in the dark, transferred from the quartz to a glass cover under light filtered by yellow cellophane, and observed under the microscope by means of light filtered through dilute copper sulfate, to remove the infrared, and by Corning filter No. 3482, to absorb wavelengths below 5350 Å. In order to test for photorecovery, other preparations were irradiated in the light, transferred from quartz to glass cover slips in white light, and kept in the unfiltered light (except for copper sulfate) of a ribbon filament research lamp that was focussed by the condenser of the microscope to illuminate the whole tissue mass. During each observation period, which required 2 to 3 minutes, the light was restricted to the individual cells being examined.

Monochromatic ultraviolet radiation was obtained with a quartz monochromator. The radiation source was a medium pressure mercury vapor lamp (6). The ultraviolet beam emerging from the monochromator was bent downward by a right-angle reflecting quartz prism situated at the exit slit. A quartz lens just below the prism focussed the image of the exit slit in the plane of the material to be treated. Before each exposure the intensity of the desired wavelength was measured at the site of treatment with a thermopile connected to a high-sensitivity galvanometer. These

had previously been calibrated against a U.S. Bureau of Standards lamp. The absorbing surface of the thermopile which measured about 1.0 × .1 cm, was slightly smaller than the image of the exit slit; therefore the whole absorbing surface lay inside the spectral line. The intensity used was the average of several measurements. Corrections were made for reflection by the surfaces of the quartz cover under which the hanging drop was suspended, so that the doses used represent the amount of radiation actually reaching the cells.

Probably the main source of error in determining the amount of radiation received by the tissues was the variation in intensity of the spectral line from end to end as the result of an uneven coating that develops in the lamp during use. To correct in part for this the embryonic tissues, which measure about .1 cm in width and .2 cm in length, were moved back and forth from end to end of the measured portion of the line during the period of treatment.

The results shown in Table I are expressed in terms of the 50 per cent or median effective dose, that is, the dose required to produce spheration of at least one of the nucleoli in 50 per cent of the cells observed for each of the wavelengths studied. This was calculated by probit analysis for each wavelength from the relation of dose to per cent of interphase and prophase cells affected. In the action spectrum shown in Fig. 3, the points represent the reciprocals of the energies required to produce the given effect, the scale being chosen so as to give the value 100 to the effectiveness of the most efficient treatment. Between five and thirty embryos were used at each wavelength. Usually ten to twenty neuroblasts were examined in each embryo for the nucleolar effect. Observations were made at 10- to 15-minute intervals following treatment until recovery began to appear. In calculating the 50 per cent dose the maximum per cent of cells affected at any one time interval after irradiation was used.

OBSERVATIONS AND RESULTS

1. *Nucleolar Spheration*

The sequence of nucleolar changes taking place during the mitotic cycle of the normal neuroblast is shown in Fig. 1. The two nucleoli of the untreated neuroblast arise in middle telophase in the region of the secondary constrictions near the distal ends of one of the longer pairs of chromosomes. They are at first highly refractile spheres that increase in size until they are 3 to 4 μ in diameter. During late telophase they gradually acquire an irregular shape and lose much of their highly refractile character. In this form they persist until very late prophase, when, shortly before their disappearance, they reassume the spherical form and their refractility increases.

If the nucleoli are irradiated and examined between telophase and their disappearance at late prophase, each is seen to break up into a group of about ten nucleolar fragments (Fig. 2A), which gradually separate and form very refractile spherules (Fig. 2B and 2C). These gradually fuse to form one large sphere (Fig. 2D–2F) that disappears at late prophase. If it per-

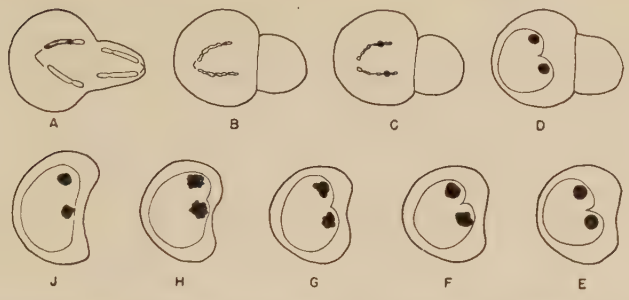


Fig. 1. Mitotic history of the neuroblast nucleolus. A: late anaphase, showing the pair of chromosomes that will develop nucleoli; nucleolar organizers are at chromosome constrictions. B: early telophase; chromosomal constrictions have not yet developed nucleoli. C: middle telophase, nucleoli have just appeared as small vesicles at the chromosomal constriction. D and E: middle telophases, showing increase in size of spherical nucleoli. F and G: late telophase; nucleoli are no longer spherical. H: interphase; nucleolus highly irregular in form. J: very late prophase, just before breakdown of the nuclear membrane; nucleolus becoming smoother in outline.

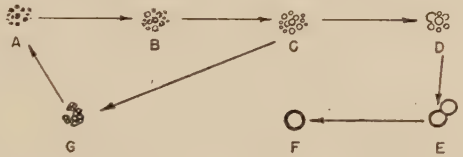


Fig. 2. Progressive changes undergone by a nucleolus subjected to a large dose of ultraviolet radiation. A and B: fragmentation. C: spheration. D–F: successive stages in the fusion of spherules to form one large sphere. G: recovery stage, in change from C to normal.

sists until after the breakdown of the nuclear membrane, however, as happens after large doses, it appears to dissolve in the cytoplasm (4). The above sequence of changes may be interrupted by recovery and return to an apparently normal condition before the later changes occur, if the dose is not too large. Thus very small doses cause fragmentation, which is followed shortly by fusion and return to normal, irregular shape; larger doses result in the formation of clusters of spherules, which may fuse (Fig. 2G) and return to the normal form either before or after losing their spherical shape; very large doses result in the formation of a sphere that persists. The appearance of clusters of nucleolar fragments of spherical shape and high refractility was chosen in the present study as the criterion of positive effect of ultraviolet radiation, and their disappearance as an indication of recovery.

If the cell is treated at prometaphase, metaphase, or anaphase, the nucleolar material appears at telophase in the form of many very small spherical bodies scattered throughout the nucleus.

Table I

Effectiveness of different wavelengths of ultraviolet radiation in producing nucleolar spheration in 50 per cent of the interphase and prophase cells examined.

Wavelength in Å	Intensity Range ×115.7 ergs/cm²/sec	No. of Cells		Median Effective Dose			Probability of Observed Difference
		Light	Dark	Light	Dark	Difference	
2 250	.15— .30	60	71	26 300	23 010	3 290	.21
2 399	.15— .60	70	130	4 487	4 375	112	.97
2 537	2.3 —12.1	51	92	3 475	3 112	363	.88
2 650	1.2 — 7.2	165	255	2 213	1 766	447	.005
2 804	.8 — 4.2	50	84	3 177	2 004	1 173	[1]
3 022	3.0 —25.9	50	51	80 170	61 660	18 510	.0004
3 130	16.0 —73.0	57	53	[1]	2 178 000	[1]	[1]

2. *Photorecovery*

The median effective doses of all wavelengths studied were consistently higher for cells kept in the light after ultraviolet radiation than for those kept in the dark (Table I, Fig. 3). The probability of the observed difference of these values for the different wavelengths indicates that at 2250, 2399, and 2537 Å exposure to visible light has no significant effect on the recovery process. At 2650 and 3022 Å, however, the differences are highly significant, and it is likely that 2804 and 3130 Å would also show a significant difference, if the data were sufficient to make possible the determination of the standard errors involved.

Posttreatment does not affect the time of appearance of nucleolar spheration, but only the extent to which it appears. Photorecovery takes place, therefore, during the time when some physical or chemical change in or adjacent to the nucleolar substance is present, but before this alteration is reflected in the morphology of the nucleolus.

3. *Effectiveness of Different Wavelengths*

Examination of the action spectrum shown in Fig. 3 shows that wavelengths 2650 and 2804 Å are most effective in producing spheration of the nucleolus. Above 2804 Å the effectiveness of ultraviolet radiation decreases until at 3650 Å no spheration effect is obtained, even after 262 000 000 ergs/cm². This closely parallels the extent to which these higher wavelengths are absorbed by nucleic acid and protein. Below 2650 Å the wave-

[1] Data insufficient to compute these values.

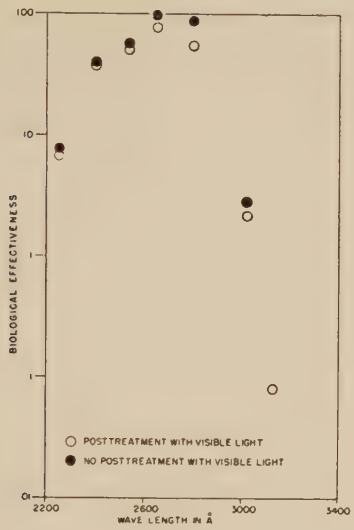


Fig. 3.

lengths also become progressively less effective. In this region the absorption by nucleic acid at first diminishes, then increases at 2250 Å. The absorption by protein of this wavelength, however, is so high that most of the energy is apparently filtered out by the overlying protoplasm and does not reach the nucleoli.

4. *Time of Appearance of Spheration*

The time interval between the termination of ultraviolet treatment and maximum spheration, as determined by the per cent of cells affected, is independent of wavelength and dose. At all wavelengths between 2250 and 3130 Å, if the dose is sufficient to induce spheration, it rarely makes its appearance in less than 20 or in more than 50 minutes; in the majority of cells it appears between 25 and 35 minutes after treatment.

5. *Duration of Spheration*

Spheration persists for periods of time varying directly with the per cent of cells affected and appears to be independent of wavelength. For example, if one or both nucleoli in 50 per cent of the cells are affected, recovery will have taken place about 35 minutes later, whether they were exposed

Table II

Percentages of cells showing nucleolar spheration in different mitotic stages after exposure to different wavelengths of ultraviolet. Data for all doses except those producing 0 and 100 per cent effect are lumped together. Numbers of cells tested are in parentheses.

Wavelength in Å	Interphase	Very Early Prophase	Early Prophase	Middle Prophase	Late Prophase
2 250	68 (28)	64 (25)	49 (53)	50 (20)	33 (3)
2 399	73 (22)	57 (23)	57 (56)	39 (26)	33 (3)
2 537	63 (92)	45 (98)	38 (108)	24 (34)	—
2 650	67 (90)	68 (92)	56 (113)	52 (35)	37 (19)
2 804	64 (22)	18 (17)	27 (30)	22 (9)	22 (9)
3 022	73 (11)	42 (12)	40 (15)	22 (9)	—
3 130	45 (9)	22 (9)	28 (25)	20 (10)	—

to 62 000 ergs/cm² of wave length 3022 Å or to 1800 ergs/cm² of wavelength 2650 Å. Abnormalities produced by a 10 per cent dose disappear in about 7 minutes, regardless of what wavelength was used to produce the spheration. Beyond the minimum dose needed to affect 100 per cent of the cells, doses can be given that prevent recovery for the several hours during which the cells can be kept under observation.

6. *Sensitivity of Different Mitotic Stages*

The sensitivities of nucleoli in cells of different mitotic stages exposed to different wavelengths of ultraviolet radiation are shown in Table II. Though the data are not very extensive, it can be seen, that, in general, interphase is the most sensitive, followed by very early prophase, early prophase, middle prophase, and late prophase, in decreasing order of sensitivity.

We have no evidence at present to indicate whether this represents a gradual loss of sensitivity by changes within the nucleolus as mitosis progresses or only a progressive decrease in the amount of incident radiation reaching the nucleolus as a result of increased absorption in the overlying portion of the cell.

7. *Mode of Action of Radiation*

In order to determine whether the spheration effect is the result of direct action of the radiation on the nucleolus or of indirect action through a primary effect on the cell or its nucleus, the relative effectiveness of the radiation in inducing spheration in nucleoli situated at different levels in the nucleus

was studied. It was found that, occasionally, only one of the two nucleoli in a nucleus underwent spheration. In the great majority of these cells the nucleolus nearer the cover glass was the affected one. While this does not demonstrate conclusively that the site of action of the treatment is restricted to the nucleolus itself, it does indicate that, if the effect is indirect, it must be brought about through changes induced in the nuclear material in the immediate vicinity of the nucleolus and probably not from diffusible substances produced at some distance. The rare cases in which the lower nucleolus only was affected might have been due either to movement of the nucleus or nucleolus after irradiation or to a local concentration of ultraviolet-absorbing materials over the upper nucleolus.

8. *Effects of Other Agents*

An effect morphologically similar to that produced with ultraviolet can be produced by heat treatment. Exposure of embryos to 44° C for 5 minutes produced nucleolar abnormalities in about 50 per cent of the cells. In contrast to the uniformity from cell to cell of the ultraviolet-induced effect, however, different cells show different degrees of spheration and separation of the nucleolar spherules. This heat-induced alteration of the nucleolus is only superficially, and not basically, identical with the ultraviolet effect. First, the spherules produced by heat are in general smaller and more numerous and form a more compact group than those produced by ultraviolet. Second, the heat-induced abnormalities appear immediately after treatment and disappear about 15 minutes later, whereas ultraviolet spheration does not become evident until about 30 minutes after treatment. Third, there is no additive effect of ultraviolet and heat treatment. If heat treatment precedes or follows immediately 2804 Å ultraviolet radiation, the heat induced nucleolar abnormalities will have appeared and disappeared by the time the ultraviolet-induced ones appear; if a 50 per cent dose of ultraviolet is followed 20 minutes later by a 50 per cent dose of heat, so that the two effects are presumably superimposed in time, there is no appreciable increase in the proportion of cells which would be affected by either agent alone.

A dose of 10 000 r of X rays administered to an embryo causes slight nucleolar abnormalities in a few cells, while the nucleoli of the remainder are apparently unaffected. Newly formed nucleoli in telophase cells seem to be most susceptible. Occasionally nuclei which appear to be in late telophase and which should, therefore, contain two nucleoli irregular in outline, contain instead from two to six spherical nucleoli. This effect is ap-

parently quite different from that produced by ultraviolet irradiation of telophase nuclei. Nucleoli which had already lost their spherical shape when X-rayed were usually unaffected. Occasional cells with fragmented nucleoli resulting from X radiation differed in their lack of spheration from those induced by ultraviolet.

Exposure of eggs to near infrared (8 000 to 15 000 Å) continuously for as long as 5 days failed to produce any visible morphological change in the neuroblast nucleoli.

DISCUSSION

The results of X-raying the nucleoli of amphibian eggs with 50 000 and 60 000 r have been reported by Duryee. His photographs (8) show a definite spheration of the nucleoli, which in that cell are numerous and adhere to the nuclear membrane. He also found (9) fusion of nucleoli after X-raying and after treatment with hypertonic (0.5 M) NaCl, which he interprets as resulting from a surface change in the nucleoli permitting their coalescence. Fragmentation of the nucleoli could be produced by the slow addition of hydrating reagents or hypotonic (0.02–0.05 M) NaCl. A striking feature of these nucleoli, which we have not seen in neuroblast nucleoli treated with ultraviolet, is the highly vacuolated nature of the interior. In this respect they resemble closely the ultraviolet-induced bodies described for the macronucleus of *Paramecium aurelia* by Kimball (11). He suggests that these bodies in *Paramecium* may consist of nucleolar material. Photographs in a recent paper by Kimball and Gaither (12) show them as rounded, highly refractile bodies not unlike the X-ray-treated amphibian nucleoli or the ultraviolet-irradiated neuroblast nucleoli.

Caspersson and Schultz (5), using ultraviolet absorption measurements, demonstrated that nucleoli of a variety of organisms contain both nucleic acid and protein. Techniques using ribonuclease in conjunction with stains have shown the nucleic acid to be of the ribose type (15). Pollister (unpublished) has calculated the ratio of protein to nucleic acid concentration in the nucleolus of *Zea mays* as 36.0 : 3.5. Owing to the much higher specific absorption of nucleic acid than protein, however, one would expect the absorption by nucleic acid to be at least equal to that by protein at the longer wavelengths, despite the much larger protein content of the nucleoli. Since our action spectrum of nucleolar spheration shows a maximum effectiveness of the 2650 and 2804 Å lines, it would be logical to conclude that nucleolar spheration can be produced by ultraviolet-induced alterations of either the nucleic acid or protein of the nucleolus. The fact, however, that we have

not been able to observe any differences in the nucleolar spheration induced by different regions of the ultraviolet spectrum, with respect to either microscopic appearance or time of maximum development, suggests that we are dealing with an effect induced in a single constituent of the nucleolus, namely, either the nucleic acid or protein. We have not sufficient evidence to resolve this apparent contradiction at the present time.

Ultraviolet-induced changes produced by several different wavelengths have now been shown capable of reduction by subsequent exposure to visible light. Using wavelength 2537 Å, Kelner (10) and Novick and Szilard (14) demonstrated photoreactivation in bacteria; Dulbecco (7), in bacteriophage; and Novick and Szilard (14) and Marshak (13) showed that the mutation rate in bacteria and the cleavage delay in *Arbacia*, respectively, produced by this wavelength were decreased by posttreatment with visible light. Kimball and Gaither (12) found that the reduction in vigor after autogamy induced by 2650 Å was diminished by exposure to visible light. None of these workers has extended the study of these effects to the longer wavelengths of ultraviolet radiation, so it is not known whether or not the wavelengths used, which are highly absorbed by nucleic acids, give maximal photoreactivation. In *Paramecium aurelia*, Kimball and Gaither (12) tested wavelengths 2378, 2537, 2650, and 2804 Å, and found a significant effect of visible light in reducing retardation of cell division after 2804 Å; after the three shorter wavelengths light appeared to decrease the effect of the ultraviolet, though the significance was questionable. They also found that visible light decreased the effects of 2804 Å in producing death before autogamy and fusion of macronuclear granules; other wavelengths were not tested for these effects.

In our studies of nucleolar spheration, effects produced by the shorter wavelengths, 2250, 2399, and 2537 Å, gave little indication of photorecovery, while 2650 and 3022 Å gave significant photorecovery. It appears that photorecovery is manifest after 2804 and 3130 Å also, but we do not have extensive enough data at these wavelengths to make a biometrical determination of significance. It seems puzzling that a certain structural change, such as nucleolar spheration, should undergo photerecovery if induced by certain wavelengths but not if brought about by certain others, especially when the alterations produced by them are to all appearances identical. Further investigations utilizing a variety of ultraviolet wavelengths are needed to clarify this situation, that is, to indicate whether the biological effect produced or the wavelength of ultraviolet radiation causing it determines the degree to which photorecovery may occur.

SUMMARY

1. Spheration induced in the nucleous of the grasshopper neuroblast by wavelengths 2250, 2399, 2537, 2650, 2804, 3022, and 3130 Å was studied.

2. Photorecovery was not evidenced after treatment with 2250, 2399, or 2537 Å. It was highly significant after 2650 and 3022 Å, and probably could have been demonstrated after 2804 and 3130 Å if the data had been more extensive.

3. Wavelengths 2650 and 2804 Å are the most effective ones in inducing nucleolar spheration. Above 2804 Å and below 2650 Å the effectiveness of the ultraviolet gradually decreases. At 3650 Å no effect was obtained even after prolonged exposure to radiation of high intensity.

4. The time interval between the termination of ultraviolet treatment and maximum spheration, as determined by the per cent of cells affected, is independent of wavelength and dose.

5. Spheration persists for periods of time varying directly with the per cent of cells affected and appears to be independent of the dose.

6. Interphase nucleoli are the most sensitive followed by very early prophase, early prophase, middle prophase, and late prophase, in order of decreasing sensitivity.

7. Spheration appears to result from direct action of the radiation on the nucleolus or on the material immediately surrounding it.

8. The effects produced on the nucleolus by ultraviolet, heat, and X-rays resemble each other only superficially.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Mr. Jack Moshman of the Mathematics Panel of the Oak Ridge National Laboratory for an extensive biometrical analysis of the data included in this paper.

REFERENCES

1. Carlson, J. Gordon, *Biol. Bull.*, **90**, 109 (1946).
2. Carlson, J. Gordon, and Hollaender, A., *J. Cellular Comp. Physiol.*, **23**, 157 (1944).
3. —— *Ibid.*, **26**, 165 (1945).
4. —— *Ibid.*, **31**, 149 (1948).
5. Caspersson, T., and Schultz, J., *Proc. Natl. Acad. Sci.*, **26**, 507 (1940).
6. Daniels, F., and Heidt, L. J., *J. Am. Chem. Soc.*, **54**, 2381 (1932).
7. Dulbecco, R., *Nature*, **163**, 949 (1949).
8. Duryee, W. R., *J. Natl. Cancer Inst.*, **10**, 735 (1949).
9. —— *Ann. N. Y. Acad. Sci.*, **50**, 920 (1950).
10. Kelner, A., *Proc. Natl. Acad. Sci.*, **35**, 74 (1949).
11. Kimball, R. F., *Anat. Rec.*, **105**, 543 (1949).
12. Kimball, R. F., and Gaither, N., *J. Cellular Comp. Physiol.*, **37**, 211 (1951).
13. Marshak, A., *Biol. Bull.*, **97**, 315 (1949).
14. Novick, A., and Szilard, L., *Proc. Natl. Acad. Sci.*, **35**, 591 (1949).
15. Pollister, A. W., and Leuchtenberger, Cecile, *Nature*, **163**, 360 (1949).

A SIMPLE TECHNIQUE FOR MAINTAINING MAMMALIAN EPIDERMAL MITOSIS *IN VITRO*

W. S. BULLOUGH[1] and MONICA JOHNSON

University of Sheffield, Great Britain

Received December 18, 1950

INTRODUCTION

In a series of studies of epidermal mitosis *in vivo* it has been shown that significant quantities of glucose and phosphate must be present before a mitosis can develop (1, 2), while studies of mitosis *in vitro* have indicated that oxygen is also of primary importance (5, 6). It now appears certain that these various substances are involved only at the onset of a division, which is then capable of completion in their absence (3).

To examine this question in greater detail, and in particular to distinguish more clearly between the conditions necessary for the initiation of mitosis and those necessary for its completion, it seems desirable to carry out further experiments *in vitro*. The aim of the present investigation has been to determine the simplest culture conditions in which mitosis will continue actively, emphasis being laid on the development of a suitable saline medium. When attempting to define the factors affecting mitosis the use of a medium of known composition is obviously preferable to that of a medium containing serum or embryo extract.

MATERIALS AND METHODS

The epidermis used was from the ears of Strong's CBA or Kreyberg's white label male mice of between 3 and 10 months of age. No differences in response to the experimental conditions were found between the two strains. In well fed males the epidermal mitosis rate is normally subject only to diurnal fluctuations associated with exercise and sleep. In contrast, the epidermal mitosis rate of the adult female is considerably complicated by the oestrous cycle, while that of immature mice has not yet been fully investigated.

The animals were killed by pulling their necks, and the thin peripheral regions of the ears were cut into small pieces (approximately 2.5 × 5.0 mm) by means of a

[1] Sorby Fellow of the Royal Society.

razor-sharp scalpel. It may be noted that in preparing and handling these ear fragments normal biochemical standards of cleanliness were maintained, but no attempts were made to develop an aseptic technique. Since the experiments lasted at most for five hours there was no time for the development of any significant bacterial infection.

The ear fragments were cultured in Warburg flasks. Each flask held as many as six fragments in 4 ml culture solution, and it was maintained at 38° C and gently rocked. The various solutions and gas phases used in the experiments are described below.

The fragments of ear were finally fixed in Bouin's alcoholic fluid. Since the mouse ear is so thin and contains such tough sheets of cartilage and connective tissue, sectioning presents a special problem. With a heavy rotary microtome normal techniques can be used, but with a rocking microtome it has proved best to embed the ear fragments as follows. After 12–24 hr in the fixative, transfer for 3 hr to 70 per cent alcohol, and for 6 hr to 95 per cent alcohol. Then leave for 12 hr (or overnight) in a half and half mixture of 95 per cent alcohol and cellusolve, for 9 hr in cellusolve, and for 12 hr (or overnight) in a half and half mixture of cellusolve and ester wax (8) in an embedding oven. Finally soak for 6 hr in pure ester wax in the oven.

When casting the blocks, each ear fragment should be picked from the ester wax, and any surplus wax clinging to it drained off against the side of the vessel. It can then be embedded in paraffin wax and cut in the usual manner. The hard ester wax provides the support while the surrounding paraffin wax makes for easy ribboning. It is, however, important that the surplus ester wax should be drained from the ear surface for, if it remains as a thin sheath, difficulties may arise when the sections are floated out due to the different melting points of the two waxes.

The sections were stained in Ehrlich's haematoxylin and eosin. As in previous work, mitotic activity was estimated by counting the total numbers of mitoses in all phases of division in unit lengths of 1 cm of ear epidermis cut 7 μ thick. Ten such counts were obtained from each ear fragment, and an average was taken. From the average figures obtained in this way in each experimental group, a grand average and standard error was calculated by the method recommended for small samples by Simpson and Roe (7).

OBSERVATIONS

1. *The epidermis*

In devising suitable experimental conditions it was necessary to check that the size of the ear fragment used was not so large as to prevent adequate penetration by the nutrients in the medium. Mouse ear epidermis is very thin, and with simple staining techniques it is normally divisible only into a Malpighian layer, some two or three cells thick, and an outer cornified layer. The name stratum germinativum must be reserved for the single basal layer of Malpighian cells adjacent to the dermis, since it is only these cells which divide.

A number of factors may have been involved in determining the penetration of the various nutrients to this stratum germinativum. Presumably penetration was easiest through the cut edges, but if this had been the only point of entry a gradient in mitosis distribution might have appeared. In fact no such gradient was observed, and it seemed that penetration was relatively uniform over the whole surface of the ear fragment. Each ear fragment, about 2.5 × 5.0 mm in area, varied in thickness from about 0.4 mm to about 0.2 mm at the ear edge. However, it was obvious that the most significant distance was that separating the cells of the stratum germinativum from the culture medium. This varied from about 10 to 40 μ, with an average of about 15 to 20 μ. The only resistance to penetration by this route seemed to be the sebaceous secretion on the skin surface, but evidently at 38° C this secretion melted and was washed away in a few minutes.

With regard to the diffusion of oxygen, the calculation can be made that in a homogeneous tissue with a uniform oxygen uptake a thickness of 40 μ can cause little or no reduction in oxygen tension (4). Thus the development of partial anaerobiosis in the stratum germinativum appears improbable, and indeed the whole ear fragment, though it is far from homogeneous, is so thin that the oxygen tension in the central connective tissue may also be very nearly that of the outside medium.

Thus, in the experiments described below, all the indications were that a relatively rapid and uniform penetration of the various nutrients and gases occurred, and that within the first hour an equlibrium was established.

2. *The culture medium*

After experiments with a variety of saline media, it was found that good results could be obtained with the "Krebs-Ringer-bicarbonate" and the "Krebs-Ringer-phosphate" of Umbreit, Burris, and Stauffer (9). A representative experiment is summarised in table I. This records the mitosis rates of ear fragments taken from six adult male mice killed at 14.00 hr when they were asleep with a relatively high rate of epidermal mitotic activity. In two of the experiments the ear fragments were incubated in the saline solutions alone, while in the others 4 ml of an isotonic solution of glucose (0.3 M) were added to each 100 ml of the solutions.

The results obtained from the bicarbonate and phosphate buffer solutions differed little. They confirm that those mitoses present when ears are separated are able to pass to completion in an apparently normal manner, and that only in the presence of glucose do significant numbers of new mitoses

TABLE I

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of the epidermis of ear fragments incubated at 38° C in various solutions. Each figure is the average of six observations.

Time of day	Krebs-Ringer-bicarbonate				Krebs-Ringer-phosphate			
	Without glucose		With glucose		Without glucose		With glucose	
	Nos. of mitoses	Phases of division	Nos. of mitoses	Phases of division	Nos. of mitoses	Phases of division	Nos. of mitoses	Phases of division
14.00	6.2±0.36	PMAT	6.2±0.36	PMAT	6.2±0.36	PMAT	6.2±0.36	PMAT
15.00	2.4±0.18	MAT	3.7±0.17	pMAT	2.6±0.20	MAT	3.6±0.22	pMAT
16.00	0.8±0.20	p T	1.2±0.14	pmaT	0.6±0.07	p T	0.8±0.06	pmaT
17.00	0.2±0.05	pm	1.9±0.31	pmat	0.1±0.01	pm	2.6±0.17	pmat
18.00	0.4±0.08	pmat	3.2±0.15	pmat	0.3±0.10	pmat	0.8±0.12	pmat
19.00	0.1±0.06	pmat	1.2±0.18	pmat	0.2±0.05	pmat	0.2±0.05	mat

In the phases of division P represents the prophase, M the metaphase, A the anaphase and T the telophase; the capital letters refer to mitoses in progress at the time of the animals' death while the small letters refer to newly developed mitoses.

develop. It seems probable that the drop in the mitosis rate occurring in all groups during the first two hours was due to the time taken for the cells to adjust themselves fully to the new conditions.

In subsequent experiments a phosphate buffer was used exclusively, and a standard solution was devised as follows:

0.90 per cent NaCl	100	2.11 per cent KH_2PO_4	3
1.15 » » KCl	4	3.82 » » $MgSO_4$, $7H_2O$	1
1.22 » » $CaCl_2$	3	1.30 » » $NaHCO_3$	3

To 9 parts of this mixture add 1 part of 0.1 M phosphate buffer pH 7.4, made by dissolving 17.8 gm Na_2HPO_4, $2H_2O$ in 20 ml normal HCl, diluted to 1 litre with distilled water.

The possible use of colchicine in these culture experiments was next investigated. Fragments of ear from six adult male mice killed at 14.00 hr were incubated at 38° C in the standard solution described above to which were added 4 ml per cent of isotonic glucose. The results are given in table II.

It appears that about an hour elapsed before the colchicine began to exert its characteristic action of arresting mitosis in the metaphase. Consequently those mitoses already present in the ear epidermis when it was taken from the mice remained unaffected and passed normally to completion. It was the new mitoses, developing from about 15.00 hr onwards, which were affected. However, in later experiments, to ensure that none of the original

TABLE II

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of the epidermis of ear fragments incubated at 38° C in the standard phosphate buffered saline with added glucose. Each figure is the average of six observations.

Time of day	Without colchicine		With 0.1 mg colchicine per 25 ml solution		With 0.2 mg colchicine per 25 ml solution	
	Nos. of mitoses	Phases of division	Nos. of mitoses	Phases of division	Nos. of mitoses	Phases of division
14.00	6.5±0.29	PMAT	6.5±0.29	PMAT	6.5±0.29	PMAT
15.00	3.9±0.25	MAT	2.6±0.20	MAT	2.9±0.18	PMAT
16.00	1.0±0.05	PMAT	1.0±0.12	PM	0.7±0.06	PM
17.00	2.5±0.13	PMAT	2.3±0.27	PM	1.4±0.16	PM
18.00	0.8±0.12	MAT	2.2±0.21	M	1.6±0.22	M
19.00	0.5±0.08	AT	2.3±0.25	M	1.6±0.17	M

In the phases of division P represents the prophase, M the metaphase, A the anaphase and T the telophase.

mitoses were arrested, the colchicine was put into the side arm of the Warburg flask and was tipped into the main vessel after an hour's incubation.

It also appears that colchicine, used in a dilution of 0.1 mg per 25 ml culture medium, did not significantly affect the number of new mitoses developing after 15.00 hr. In the control group the maximum average number of new mitoses seen was 2.5 ± 0.13 at 17.00 hr, while with colchicine the final figure obtained was 2.3 ± 0.25. However, with double this concentration of colchicine there was a marked reduction in the numbers of new mitoses so that a final figure of only 1.6 ± 0.17 was recorded.

At this point steps were taken to discover the optimum concentration of glucose for maintaining mitosis. Six adult male mice were killed as before at 14.00 hr, and their ear epidermis was incubated at 38° C for five hours. The control group received no glucose, while the experimental groups received from 2 ml to 16 ml of isotonic (0.3 M) glucose per 100 ml saline solution. In all cases 0.1 mg colchicine was added to each 25 ml of the final mixtures, and the numbers of arrested metaphases seen after five hours are shown in table III.

From this experiment it appears that a concentration of 8 ml 0.3 M glucose per 100 ml standard phosphate buffer solution is approximately the optimum for the induction of epidermal mitosis *in vitro*. With a higher concentration of glucose a lower mitosis count was obtained, and it may be recalled that an excess of glucose also has a depressing effect *in vivo* (1).

Experiments were also carried out to test the effectiveness of serum as a

TABLE III

Average numbers of mitoses arrested by colchicine in unit lengths (1 cm) of sections (7 μ thick) of ear epidermis incubated at 38° C for five hours in standard phosphate buffered saline with added glucose. Each figure is the average of six observations.

Concentration of 0.3 M glucose in ml/100 ml saline				
0 ml	2 ml	4 ml	8 ml	16 ml
0.2±0.06	1.0±0.11	2.2±0.21	3.1±0.20	2.3±0.19

TABLE IV

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of the epidermis of ear fragments incubated at 38° C in phosphate buffered saline and in horse serum, both with added glucose. Each figure is the average of six observations.

Time of day	Phosphate buffered saline with added glucose	Horse serum with added glucose
14.00	7.2±0.39	7.2±0.39
15.00	2.4±0.22	2.1±0.17
16.00	0.9±0.10	1.1±0.13
17.00	1.6±0.19	1.9±0.16
18.00	3.0±0.28	2.8±0.19
19.00	1.5±0.21	2.1±0.23

culture medium. Originally mouse serum was used, but, as this proved too expensive in mice, horse serum had to be substituted. This was prepared from blood freshly obtained from the slaughter house, and since the blood sugar level of the horse is well below the 170 mg per cent typical of the mouse, 6 ml of 0.3 M glucose were added to each 100 ml serum. Again six adult male mice were killed at 14.00 hr, and the results are shown in table IV. The controls were incubated in a mixture of 8 ml 0.3 M glucose and 100 ml standard phosphate buffered saline.

It would seem that no advantage is gained by the use of serum, the mitotic activity developing at 18.00 hr being similar in both groups.

Conclusions. The standard phosphate buffered saline described above is suitable for the maintenance of epidermal mitotic activity *in vitro* if glucose is added in the proportion indicated. This medium also has the great advantage of a known and constant composition.

Horse serum with added glucose is also a suitable culture medium, and it has the further advantage of being a natural physiological fluid. However, for the purposes of most experiments it has the overwhelming disadvantage of an unknown and inconstant composition.

TABLE V

Average numbers of mitoses present per unit length (1 cm) of sections (7 μ thick) of the epidermis of ear fragments incubated at 38° C in saline and serum media with added glucose and with various gas phases. Each figure is the average of ten observations.

Time of day	Standard phosphate buffered saline			Horse serum		
	in air	in O_2	in O_2/CO_2	in air	in O_2	in O_2/CO_2
14.00	8.1±0.44	7.7±0.41	8.0±0.45	8.1±0.44	7.7±0.41	8.0±0.45
15.00	2.0±0.19	2.1±0.24	2.0±0.22	1.4±0.18	1.9±0.19	1.9±0.16
16.00	1.0±0.13	1.9±0.23	0.8±0.08	0.9±0.10	2.0±0.21	1.8±0.23
17.00	1.3±0.17	5.3±0.37	1.7±0.15	2.0±0.17	4.8±0.47	4.7±0.49
18.00	2.9±0.31	5.4±0.55	3.5±0.24	3.0±0.19	4.9±0.20	5.3±0.37
19.00	1.5±0.12	2.4±0.15	1.6±0.15	2.2±0.26	3.4±0.36	2.0±0.14

3. *The gas phase*

At this stage the highest average number of mitoses induced *in vitro* was only about 3 per cm length of sections of epidermis cut 7 μ thick, whereas in a normal mouse the maximum average number seen during sleep is about 7 or 8. In an attempt to increase the mitosis rate *in vitro*, attention was turned to the question of the gas phase. It was recalled that Medawar (5) has suggested that the rate of cell division in rabbit epidermis *in vitro* may vary with oxygen tension over a fairly wide range, and the next step was therefore the incubation of mouse ear epidermis in high oxygen tensions. Ten adult male mice were killed at 14.00 hr, and pieces of their ears were incubated in either standard phosphate buffered saline or in horse serum, both with added glucose. The effects of air, oxygen and a 95 per cent oxygen/5 per cent carbon dioxide mixture were compared and the results are given in table V.

Evidently in both saline and serum media the high oxygen gas phase induced a considerable increase in the epidermal mitotic activity. In serum similar results were obtained with oxygen and with oxygen/carbon dioxide, and these also compared closely with the results from the saline with oxygen group. In each of these cases the mitosis rate rose to a maximum of about 5 mitoses per unit of epidermis studied after about four hours' incubation. In contrast, the ear fragments cultured in saline with an oxygen/carbon dioxide mixture gave a maximum figure of only 3.5 which was little more than that obtained in air.

Conclusions. A high oxygen tension is favourable to the development of

epidermal mitosis *in vitro*. With a saline medium pure oxygen is best, since the presence of 5 per cent carbon dioxide may depress the mitosis rate. The reason for this depression was not sought, but it may be noted that the pH of the medium remained constant at about 7.4 throughout the experiment.

With serum similar results were obtained except that the carbon dioxide effect was not evident. This may have been due to the superior absorbing and buffering powers of serum.

SUMMARY AND CONCLUSIONS

1. The main aim of the present investigation has been to devise a simple technique whereby mouse epidermis can be maintained *in vitro* for short periods and can be induced to undergo active mitosis. To avoid complications due to the oestrous cycle of the female and to the as yet unknown conditions in juvenile mice, only ear epidermis from adult male mice was used.

2. In studying the physiology of mitosis *in vitro* it is especially important to employ media of known composition, and for this reason the most valuable technique here described depends on the use of a phosphate buffered saline with added glucose and a gas phase of pure oxygen.[1] If at any time the introduction of unknown factors is unimportant then horse serum with added glucose may be used instead. These media are most conveniently held in Warburg flasks maintained at 38° C.

3. The present results are in agreement with those of Medawar (5) in stressing the importance of oxygen. Medawar showed that while rabbit epidermal cells may survive for at least a week in strictly anaerobic conditions, a lack of oxygen inhibits both cell movement and cell division. Evidently, while epidermal survival can be ensured by anaerobic glycolysis, cell movement and division depend on aerobic reactions.

4. One final comment may be made. It has been remarked that mitotic activity in the ear epidermis *in vivo* is confined to the basal stratum germinativum. It might appear possible that this form of mitosis gradient may be at least partly due to the natural advantage of the basal cells relative to the blood supply. However, when epidermis is kept *in vitro* and the supply gradient is reversed it is still only the basal cells which develop mitotic activity. Evidently the form of this gradient is determined by other factors than the nutrient supply.

---

[1] Since this paper was completed, it has been found that a still higher rate of mitosis can be induced if the saline medium contains 0.02 M sodium glutamate, which is a catalyst of the tricarboxylic acid cycle.

REFERENCES

1. Bullough, W. S., *J. Exptl. Biol.*, **26**, 83 (1949).
2. —— *J. Endocrinol.*, **6**, 350 (1950).
3. —— *Exptl. Cell Res.*, **1**, 497 (1950).
4. Höber, R., Physical Chemistry of Cells and Tissues, Philadelphia: Blakiston Publishing Co., 1945.
5. Medawar, P. B., *Quart. J. Micr. Sci.*, **88**, 27 (1947).
6. —— *Quart. J. Micr. Sci.*, **89**, 187 (1948).
7. Simpson, G. G., and Roe, A., Quantitative Zoology, New York and London: McGraw Hill.
8. Steedman, H. F., *Quart. J. Micr. Sci.*, **88**, 123 (1947).
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Tissue Metabolism, 2nd edition. Minneapolis: Burgess Publishing Co., 1949.

INACTIVATION OF *NEUROSPORA* CONIDIA BY ULTRAVIOLET RADIATION

AMOS NORMAN[1]

Subcommittee on Biophysics, Columbia University, New York, N. Y.

Received December 24, 1950

INTRODUCTION

Many organisms are inactivated by radiation at a rate which is directly proportional to the number of viable organisms. Lea (11) has emphasized that this behavior is consistent with the assumption that the inactivation of a given organism is a consequence of the transformation of a single unit within it; moreover, he suggested that the transformation is in the nature of a gene mutation. It seems reasonable to assume, therefore, that in organisms in which the genetic material is duplicated the units, whatever their nature, are also duplicated and that they continue to exhibit a kinetics of inactivation which is first order with respect to the number of these units. Such a theory was developed recently by Atwood and Norman (3) and applied to some data on the inactivation of *Neurospora* conidia by ultraviolet radiation. The number of units, as calculated from the kinetics data, showed a correlation with the number of nuclei per conidium implying that the conidial inactivation was a consequence of a change in the nuclei. However, the data were not altogether convincing and further doubt was thrown on the central role of the nuclei in the inactivation process when Atwood (2) showed that the frequency of viable nuclei among the conidia surviving a given dose of ultraviolet radiation was much higher than expected on the assumption that the nuclear inactivation was responsible for the inactivation of the conidia. The present work was undertaken, therefore, in the hope that a careful study of the kinetics of inactivation would reveal the role of the nucleus in the inactivation process and how that role is modified by the interaction of nuclei within he same cytoplasm.

EXPERIMENTAL

Neurospora crassa 1 A, which gives multinucleate macroconidia, and the mutant strain P^{em} Fl (4), which gives uninucleate microconidia, were grown at 25° C. for about

[1] Now at the Department of Zoology, Columbia University, New York 27, N.Y.

20 and 10 days respectively on minimal medium. This medium consists of Fries salts (5), 4 μg/l biotin, 1.0 per cent sucrose, and 1.7 per cent of thoroughly washed agar. The conidia were flushed out with 0.9 per cent saline, filtered through glass wool, and diluted in saline to concentrations of 10^2–10^3 conidia/ml. A three ml suspension of conidia was placed in a quartz cuvette of the type used in Beckman spectrophotometers and irradiated. In order to minimize pipetting errors, two platings of 1.0 and 1.5 ml respectively were made from the irradiated suspension in sorbose medium. The sorbose medium, in which colonial growth takes place, is identical with the minimal medium except that the sucrose is reduced to 0.1 per cent and 1.0 per cent sorbose is added. This medium was kept at 45° in order to keep the agar melted and was poured into Petri plates in which the conidial suspension was placed. After pouring, the plates were incubated for five days at 31° C and then counted. Controls consisted of platings made from the same dilution flask from which samples to be irradiated were taken; these control platings were made both before and after the irradiation. *The inability to form colonies under these conditions was used as the criterion for the inactivation of the conidia.*

A water-cooled, quartz, mercury, capillary arc of the Daniels-Heidt type provided the ultraviolet radiation. Various of the mercury arc lines were isolated with a Bausch and Lomb quartz monochromator and focused on the cuvettes which were mounted in tandem in the beam. During irradiation the conidial suspension was constantly stirred by short sections of Alnico bar magnets coated with an inert wax and driven by a commercial magnetic mixer. This mode of stirring allowed the cuvettes to be covered during the (long) irradiations and also made it possible to keep the stirring elements completely out of the beam. The beam was monitored by a 1P28 photomultiplier mounted rigidly behind the cuvettes. The output current from the photomultiplier was read directly on a microammeter and was kept below 30 microamps to prevent fatigue. The monitoring system was calibrated with a thermopile-galvanometer system and a standard lamp. All work during and after irradiation was done under low intensity yellow light in order to avoid photoreactivation.

For the photoreactivation work the conidia were first inactivated by the 254 mμ mercury resonance line from a low pressure arc (sterilamp). The intensity of this line was determined, roughly, using a uranyl oxalate actinometer. After exposure to the ultraviolet radiation the conidia were plated out as before in two platings. One plate was put directly into the incubator, the other was first exposed for thirty minutes to light from a 500 watt projection lamp. The light was first passed through an eight cm layer of a 25 g/l solution of $CuSO_4 \cdot 5H_2O$ and the plates were vigorously air-cooled during the reactivation to minimize heat effects. No such effects were detected with controls run for this purpose.

In order to count the nuclei the conidia were stained with basic fuchsin. The interfering RNA in the cytoplasm was removed with 15 per cent perchloric acid. The Feulgen nucleal reaction which is specific for DNA was also used but was found less satisfactory for this material.

Conidial dimensions were determined from measurements made on camera lucida drawings of the conidia suspended in saline. The ordinary fixatives were found to alter these dimensions considerably.

Absorption measurements were made with the Beckman spectrophotometer using

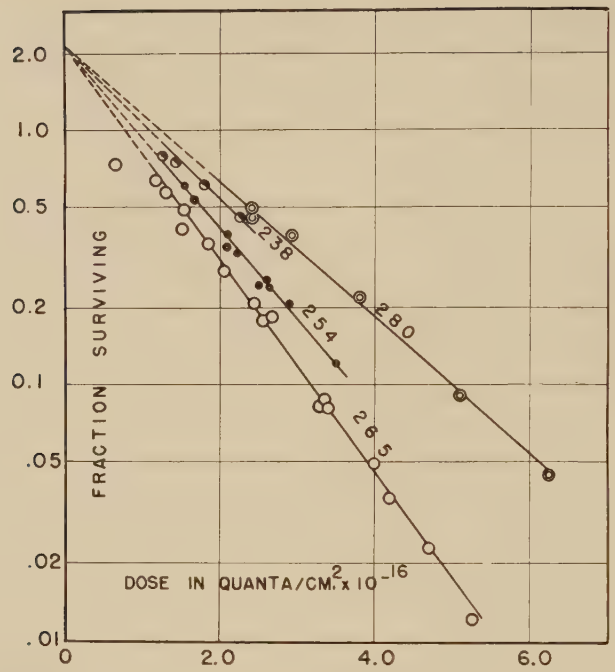


Fig. 2. Inactivation of macroconidia by ultraviolet radiation. The linear portions of the curves are extrapolated back to zero dose. The wavelengths are given in mμ.

tribution of nuclei among the macroconidia, it can be seen that the value of $\bar{n}$ is 2.27. This value is in good agreement with the average number of nuclei per conidium determined from the inactivation data. Thus, these data confirm the hypothesis that the inactivation of the conidia is a consequence of the inactivation of the nuclei and that the nuclei exhibit a kinetics of inactivation which is first order with respect to the number of (viable) nuclei.

Equations [3] and [4] imply that the fraction of conidia, S, surviving a given dose of radiation is independent both of the radiation intensity and of the initial concentration of conidia. For both conidial stocks this has been verified over a range of concentrations of 50–5 10^3 conidia/ml and over a range of intensities of 10^{11}–10^{13} quanta/cm^2 sec. The conidia were actually exposed to intermittent radiation, which imposes a more stringent test of intensity independence than these figures indicate (16). The ultraviolet beam filled only about one-twentieth of the cross-sectional area of the suspension being irradiated. The indicated range of intensities represents average values of the intensities over the total area of the suspension. Thus the conidia were exposed intermittently, as they were swept in and out of the beam by the stirrer, to intensities some twenty times the average values given.

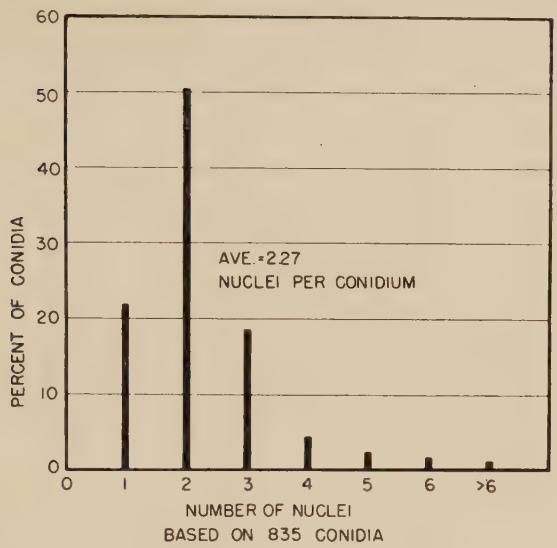


Fig. 3. Distribution of the number of nuclei per conidium among the macroconidia.

TABLE I

Inactivation Cross-Section per Nucleus in $cm^2 \times 10^{16}$.

Wavelength in mμ	238	254	265	280
Microconidia	1.14 ± .02	1.50 ± .05	1.81 ± .01	1.10 ± .02
Macroconidia	.65 ± .04	.77 ± .03	.96 ± .02	.62 ± .02

Wavelength in mμ	302
Microconidia	.082 ± .004
Macroconidia	—

The standard deviation is estimated from the scatter of the data about the straight line: $\log S = \log n - kD$. It does not include errors in the calibration of the light intensities.

B. Interaction of the nuclei. It will be noted that the inactivation cross-section, k, in both equations [3a] and [4] is the inactivation cross-section *per nucleus* in the micro- and macroconidia respectively. The nuclei in these two strains differ in only two loci; therefore it might be expected that their radiosensitivities would be about equal. Yet, a comparison of the inactivation cross-sections, given in table I, shows that the microconidia are about twice as sensitive as the macroconidia. It is tempting to ascribe the smaller cross-section (greater resistance) of the macroconidia to an interaction of the nuclei in the multinucleate macroconidia. But before this can be done two possibilities must be investigated: the difference in sensitivity may be

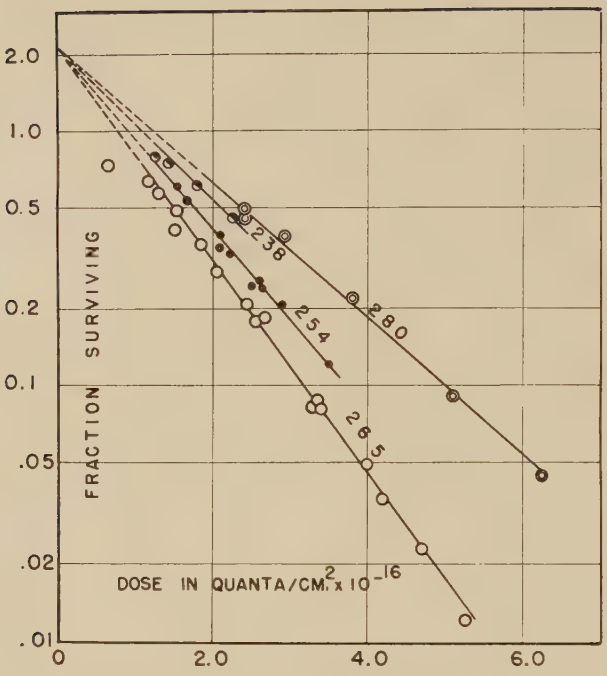


Fig. 2. Inactivation of macroconidia by ultraviolet radiation. The linear portions of the curves are extrapolated back to zero dose. The wavelengths are given in mμ.

tribution of nuclei among the macroconidia, it can be seen that the value of $\bar{n}$ is 2.27. This value is in good agreement with the average number of nuclei per conidium determined from the inactivation data. Thus, these data confirm the hypothesis that the inactivation of the conidia is a consequence of the inactivation of the nuclei and that the nuclei exhibit a kinetics of inactivation which is first order with respect to the number of (viable) nuclei.

Equations [3] and [4] imply that the fraction of conidia, S, surviving a given dose of radiation is independent both of the radiation intensity and of the initial concentration of conidia. For both conidial stocks this has been verified over a range of concentrations of 50–5 10^3 conidia/ml and over a range of intensities of 10^{11}–10^{13} quanta/cm^2 sec. The conidia were actually exposed to intermittent radiation, which imposes a more stringent test of intensity independence than these figures indicate (16). The ultraviolet beam filled only about one-twentieth of the cross-sectional area of the suspension being irradiated. The indicated range of intensities represents average values of the intensities over the total area of the suspension. Thus the conidia were exposed intermittently, as they were swept in and out of the beam by the stirrer, to intensities some twenty times the average values given.

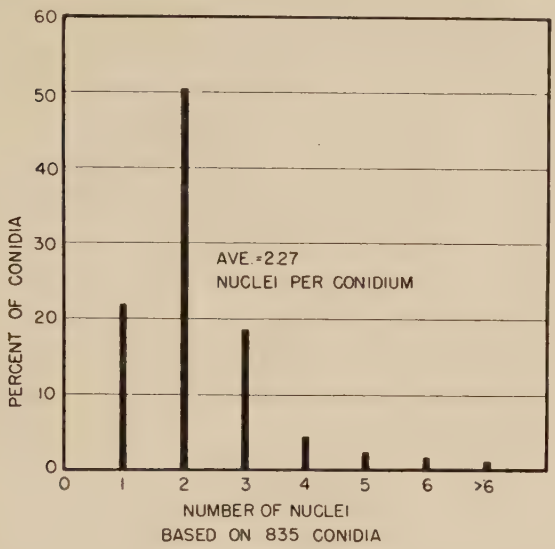


Fig. 3. Distribution of the number of nuclei per conidium among the macroconidia.

TABLE I

Inactivation Cross-Section per Nucleus in $cm^2 \times 10^{16}$.

Wavelength in mμ	238	254	265	280
Microconidia	1.14 ± .02	1.50 ± .05	1.81 ± .01	1.10 ± .02
Macroconidia	.65 ± .04	.77 ± .03	.96 ± .02	.62 ± .02

Wavelength in mμ	302
Microconidia	.082 ± .004
Macroconidia	—

The standard deviation is estimated from the scatter of the data about the straight line: $\log S = \log n - kD$. It does not include errors in the calibration of the light intensities.

B. Interaction of the nuclei. It will be noted that the inactivation cross-section, k, in both equations [3a] and [4] is the inactivation cross-section *per nucleus* in the micro- and macroconidia respectively. The nuclei in these two strains differ in only two loci; therefore it might be expected that their radiosensitivities would be about equal. Yet, a comparison of the inactivation cross-sections, given in table I, shows that the microconidia are about twice as sensitive as the macroconidia. It is tempting to ascribe the smaller cross-section (greater resistance) of the macroconidia to an interaction of the nuclei in the multinucleate macroconidia. But before this can be done two possibilities must be investigated: the difference in sensitivity may be

due to the different physiological state of the two sets of nuclei or it may be due to a difference in nuclear shielding by the cytoplasm in the two sets of conidia. The latter possibility is more easily handled and it will be dealt with first.

Let I_0 and I stand for the intensity of the incident and transmitted light, then the behavior of a conidial suspension can be represented over a wide range of concentrations, c, by the equation

[5] $$I = I_0 e^{-\epsilon cd}$$

where d is the depth of suspension traversed by the light beam and ϵ is the sum of the absorption and scattering cross-sections per conidium. Thus, in a beam of uniform intensity containing N quanta/cm² the total number of quanta which are either absorbed or scattered per conidium is given by ϵN. On the other hand, if σ is the geometrical cross-section of the conidium then σN is the number of quanta which are incident on the conidium. Therefore the ratio ϵ/σ represents the fraction of quanta incident per conidium which is scattered or absorbed and $1 - \epsilon/\sigma$ is the transmission of the conidium. In figure 4 is given a plot of ϵ as a function of wavelength. It can be seen that at 260 mμ ϵ has the value of 13.7×10^{-8} and 3.24×10^{-8} cm² for the macro- and microconidia respectively. The geometrical cross-sections are 33.8×10^{-8} and 7.95×10^{-8} cm². Thus, for each conidial type the average transmission turns out to be about 59 per cent. The close agreement is probably fortuitous since there are errors of the order of 5 per cent in the determination of both ϵ and σ. However, these measurements do show that a difference in nuclear shielding cannot account for the factor of two difference in the inactivation cross-sections of the two stocks.

The radiosensitivity of the stocks is a function of their physiological state. This is shown, for example, by the marked change in sensitivity with age of the macroconidia. Indeed, older macroconidia were used because of their increased sensitivity. Thus the factor of two in sensitivity between the two stocks is probably a minimum value as far as this one factor of age is concerned; but this does make the question even more pressing of whether the whole difference in inactivation cross-sections is not due to physiological differences in the stocks of micro and macroconidia. Now it can be seen from figure 3 that the macroconidia have a uninucleate component which comprises some 22 per cent of the stock. If the difference in sensitivity of the two stocks is due to an interaction of nuclei in the multinucleate conidia, then the uninucleate component of the macroconidia should have about twice the inactivation cross-section of the other (multinucleate) components. While

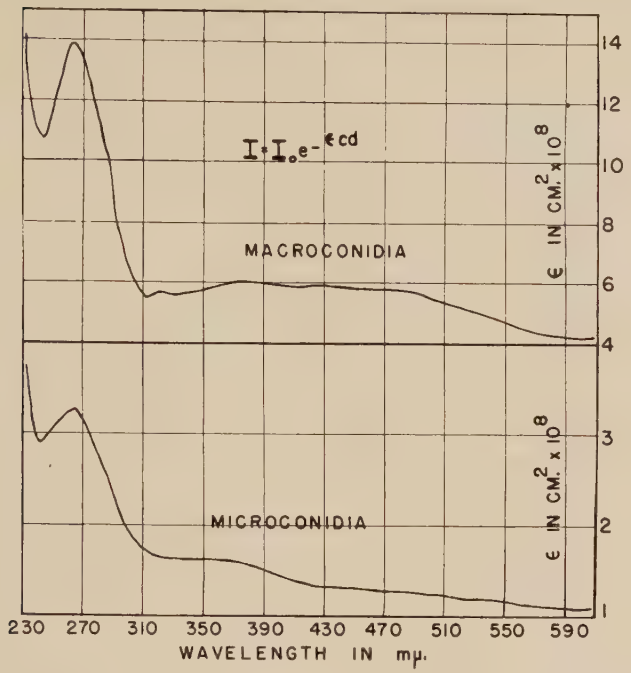


Fig. 4. Sum of the absorption and scattering cross-sections per conidium as a function of wavelength.

it has not been possible to separate this uninucleate component from the rest of the macroconidia, it is easily demonstrated that the increased radiosensitivity in this component will simply serve to lower the point determined by extrapolating back to zero dose the plot of log S vs. D to a value about 10 per cent lower than that of the average number of nuclei per conidium. In fact, the data for the inactivation of the macroconidia do extrapolate back to about 2.1 whereas the average number of nuclei per conidium is about 2.27. By applying simple linear normal regression theory to the inactivation data it is possible to test the significance of this difference of .17 which amounts to about 10 per cent of the value of the average number of nuclei per conidium. The difference is statistically significant; i.e., the probability is better than 90 per cent that the difference is not due to expected errors in the two sets of data. The possibility exists, of course, that the number of functional nuclei is less than the number of nuclei, but this is highly unlikely since in both stocks, under the conditions of these experiments, the number of conidia which can form colonies in sorbose medium agrees very closely with the

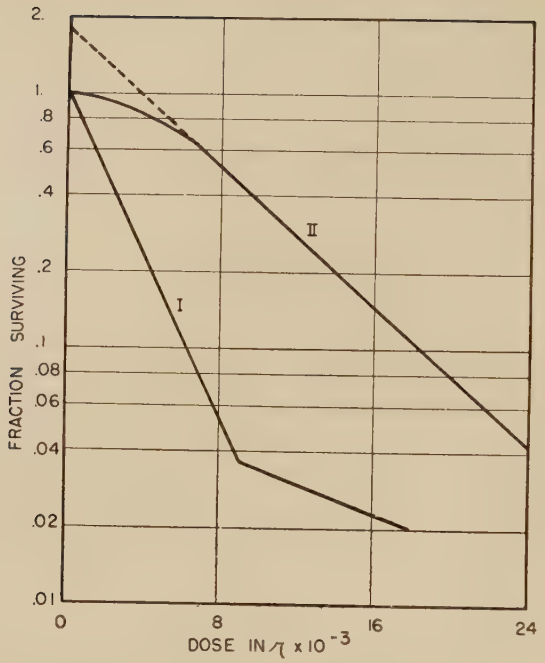


Fig. 5. The inactivation of yeast cells by X-rays. I. Haploid cells. II. Diploid cells. The data are from Latarjet and Ephrussi (10).

number of conidia plated out as determined by direct count in a hemocytometer.

The arguments advanced in the preceding paragraphs find strong support in some data of Latarjet and Ephrussi (10) which I have redrawn in figure 5. These data show that the inactivation of diploid and haploid yeast cells by X-rays is strikingly similar to the ultraviolet inactivation of macro and microconidia respectively. The plot of log S vs. D gives a straight line which extrapolates back to about 2 in the case of the diploid stock and goes through 1 in the case of the haploid stock. Moreover, the haploid stock shows about twice the radiosensitivity of the diploid stock. In the case of X-rays there is no problem of shielding and it seems very likely that the difference in sensitivity is due to an interaction of the two sets of chromosomes in the diploid yeast.

C. The Inactivation Process. *Neurospora* nuclei are haploid, the single chromosome complement consists of seven chromosomes which must contain a large number of loci. A lethal mutation at any locus will result in the inactivation of a uninucleate conidium. However, the vast majority of

ultraviolet-induced mutations are non-allelic [1] indicating that a very large number of loci mutate at about the same rate; therefore, if lethal mutations alone are responsible for the inactivation of the nuclei the multinucleate conidia would be at least an order of magnitude more resistant than the uninucleate conidia, since the probability of inducing lethal mutations in identical loci of all the nuclei in a multinucleate conidium is very small compared to the probability of inducing a mutation at any locus. It seems necessary, therefore, to ascribe the inactivation of the macroconidia almost wholly to some non-genetic process affecting the nuclei. (The term "non-genetic" applied to a lethal effect seems meaningless at first glance; but an operational definition will be given in the discussion to both the genetic and non-genetic processes.) The greater sensitivity of the microconidia is attributable, then, to the fact that both genetic and non-genetic effects are contributing to the inactivation of the uninucleate microconidia.

Since the inactivation of multinucleate conidia is ascribed to the non-genetic process, the probability that a nucleus survives this process must have the form

$$[6] \qquad S_1 = e^{-k_1 D}$$

where the subscript refers to the non-genetic process. *Assuming that the genetic and non-genetic processes are independent,* the survival of a uninucleate conidium will be given by the product $S_1 S_2$ where S_2 is the probability that a nucleus survive the genetic process. But the survival of the uninucleate conidia is given by equation [3a] and the form of this equation will be preserved, on the assumption that the two processes are independent, only if

$$[7] \qquad S_2 = e^{-k_2 D}.$$

In terms of the two processes equation [3a] can be rewritten as

$$[8] \qquad S(1) = e^{-(k_1 + k_2) D}$$

while the survival of macroconidia at high dose becomes

$$[9] \qquad S(n) = \bar{n}\, e^{-k_1 D}.$$

From equations [8] and [9] it can be seen immediately that the ratio of the inactivation cross-sections of the macro- and microconidia gives the ratio $k_1/(k_1 + k_2)$. This ratio is approximately equal to 1/2, showing that the genetic and non-genetic effects have about equal cross-sections; i.e., it is about equally probable that a nucleus will be inactivated by a lethal mutation or by some non-genetic process.

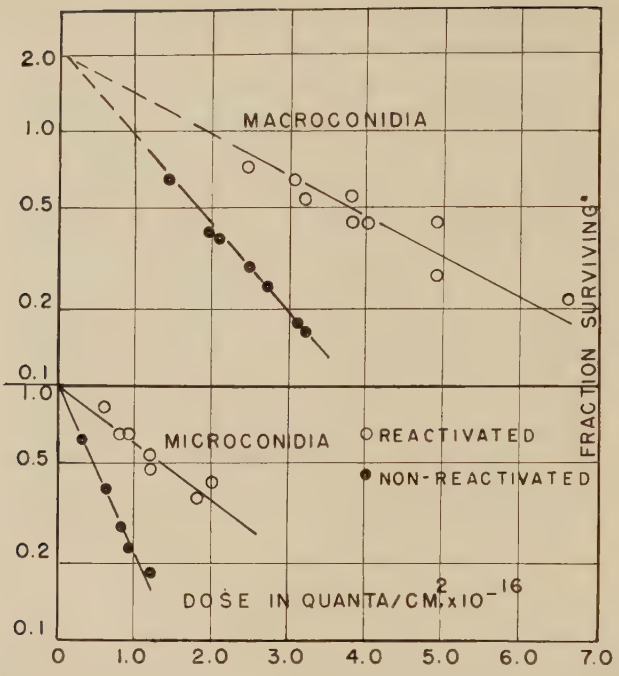


Fig. 6. The effect of photoreactivation on the survival of ultraviolet-irradiated condidia. The inactivating wavelength was 254 mμ.

D. Photoreactivation. In figure 6 are presented the data for the photoreactivation of the two stocks. It can be seen that the effect of photoreactivation can be described in terms of a reduction in the effective ultraviolet dose (15, 9) or, equivalently, in terms of a decrease in the inactivation cross-sections. Using primes to designate the inactivation cross-sections after photoreactivation, the data can be summarized by

[10] (a) $$(k_1 + k_2)/(k'_1 + k'_2) = 2.9$$

(b) $$(k_1/k'_1) = 2.1.$$

These numerical results indicate clearly that both the genetic and non-genetic effects are reduced by light-reactivation. The genetic effect seems to be reduced to the greater extent, but there are too many uncertainties connected with the photoreactivation to consider the difference significant. However, the data do indicate definitely that both the genetic and non-genetic effects of ultraviolet radiation are reduced by the same order of magnitude by pho-

toreactivation. Novick and Szilard (15) have reached the same conclusion on the basis of experiments done with *E. coli*. Since their results do not depend on the assumption that the genetic and non-genetic processes are independent whereas the analysis embodied in equation [10] does, the agreement in our conclusions lends support to the assumption that the two inactivation processes are independent. At any rate, the two processes cannot be differentiated conclusively on the basis of their response to photoreactivation.

E. Action Spectrum. The primary step in both the genetic and non-genetic process consists in the absorption of a quantum of ultraviolet radiation by some intracellular substance. The action spectrum for the inactivation of the conidia throws light on the nature of this substance. Moreover, any significant difference in the action spectrum of the micro- and macroconidia would indicate a difference in the genetic and non-genetic processes at the level of the primary photochemical act. To facilitate comparison between the two stocks their relative inactivation cross-sections have been plotted together in figure 7 and a typical nucleic acid absorption curve (6) has been superimposed for comparison. It can be seen immediately that the two action spectra are identical and that they resemble strikingly the absorption spectrum of nucleic acid. Thus, the genetic and non-genetic processes can be differentiated neither by their action spectra nor by their response to photoreactivation.

In order to calculate the number of quanta absorbed per conidium, on the average, before the conidium is inactivated, it is necessary to know the absorption spectrum of the conidia. If we designate the absorption and scattering cross-section per conidium by a and s, respectively, then we can write

$$\epsilon = a + s \qquad [11]$$

where ϵ is defined by equation (5). In general it is not possible even theoretically to separate out the contributions of the absorption and scattering in this simple fashion. In the case of the conidia, however, the bulk of the scattering occurs at the surface of the conidia due to the sharp change in refractive index between the saline solution and the conidial wall; the absorption, on the other hand, is due entirely (for all practical purposes) to the interior of the conidia. Thus the conidia can be considered as composed of two different non-interacting systems one of which is a simple scatterer, the other a simple absorber. To the extent that this model is valid equation [11] is justified.

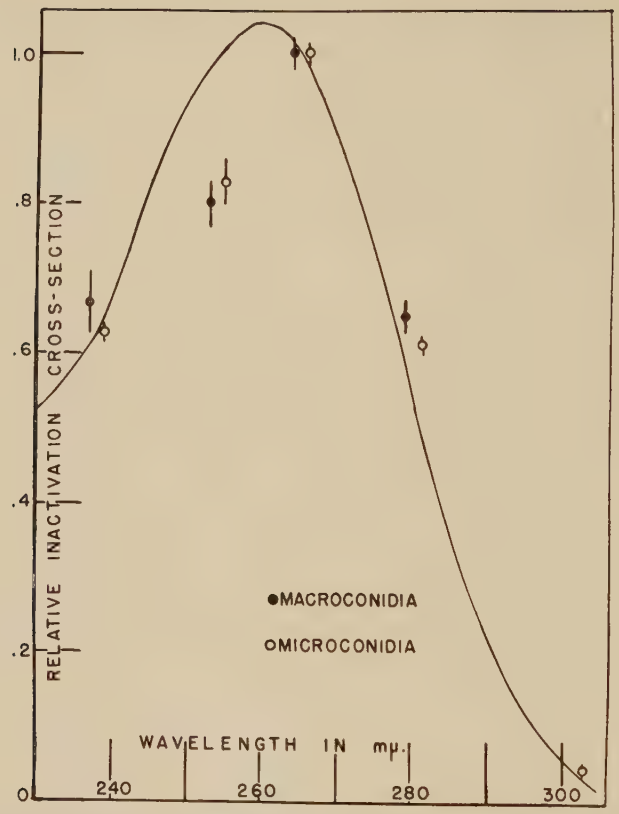


Fig. 7. Action spectra for the inactivation of *Neurospora* conidia. The experimental points have been displaced 2 mμ to either side of the corresponding wavelength to facilitate comparison. The curve is a nucleic acid absorption spectrum from Caspersson (6) drawn to give the best fit to the experimental data.

The average diameters of the macro- and microconidia measure 6.6 μ and 3.2 μ, respectively. At wavelengths shorter than 600 mμ these diameters are greater than ten and five times the wavelength of light, respectively; so that over this wavelength region geometrical optics should apply. This implies that the scattering cross-section is (roughly) independent of wavelength and that it is directly proportional to the geometrical cross-section of the conidia. As expected, the plot of ϵ, in figure 4, shows no indication of a marked wavelength-dependent scattering—for example, the nucleic acid peak at 260 mμ shows very little distortion. Indeed, the curves in figure 4 are quite similar to the *absorption* spectra of cells as obtained with a microspectrophotometer (6). At 600 mμ *Neurospora* pigments show negligible

absorption (7) and only the scattering cross-section contributes to ϵ. Again, as expected, the ratio of ϵ of the macro- and microconidia at 600 mμ is about equal to the ratio of their geometrical cross-sections. Finally, the expectation that there is little scattering from the interior of the conidia is born out by the marked lack of internal structure to be seen in photographs of conidia taken by either visible or ultraviolet light. Therefore, equation [11] is apparently justified and it seems that a good approximation to the absorption cross-section at a given wavelength shorter than 600 mμ can be made by subtracting the value of ϵ at 600 mμ from the value of ϵ at that wavelength. Obtaining the value of the absorption cross-section, a, in this fashion we can write from equations [5] and [11] that the fraction of light absorbed by a suspension is

$$[12] \qquad 1 - e^{-acd} = acd.$$

(The approximation $e^{-acd} = 1 - acd$ is excellent at the low concentrations of conidia used in the irradiation experiments.) Thus, each conidium absorbs quanta at the rate Ia. For the microconidia the fraction of viable conidia at any time is given by e^{-kIt} so that the rate at which quanta are absorbed per viable conidium is aIe^{-kIt} Therefore, the total number of quanta absorbed per conidium, on the average, before it is inactivated is given by

$$[13] \qquad \int_0^\infty aIe^{-kIt}\, dt = a/k.$$

The same results are obtained with macroconidia if the nucleus is considered as the unit of inactivation rather than the conidium. Therefore, it seems reasonable to define the efficiency of radiation as the ratio k/a, the ratio of the inactivation cross-section per nucleus to the absorption cross-section per conidium. In table II the efficiencies so defined are tabulated.

TABLE II

Radiation Efficiency $\times 10^9$.

Wavelength in mμ	238	254	265	280	302
Microconidia	6.0	7.1	8.4	6.6	1.1
Macroconidia	.93	.90	1.0	.91	—

The efficiency is defined as the ratio of the inactivation cross-section per nucleus to the absorption cross-section per conidium.

The efficiencies for the microconidia are of the order of 10^{-8} while for the macroconidia they are about 10^{-9}. These efficiencies are extremely small. The quantum efficiency for the inactivation of a typical virus is in the order of 10^{-5} [13]; the virus may be considered as a sort of "naked nucleus" and the inactivation of a nucleus should have an efficiency within one or two orders of magnitude of this quantity. Now it must be remembered that by my definition efficiency is a hybrid quantity using the inactivation cross-section *per nucleus* but the absorption cross-section per conidium. Considerations based on staining properties of cytoplasm and nucleus, on their relative volumes, and on their appearance in photographs made with the ultraviolet microscope lead to the conclusion that the nuclei contain only some 1/100 the total nucleic acid in the conidia. Therefore, if only quanta absorbed in the nucleus can lead to its inactivation and only these are used in calculating efficiency, then the efficiency of inactivation will be in the (more reasonable) order of 10^{-6}, the same figure obtained for the inactivation of *E. coli* (12). Similarly, the large difference in the efficiency of nuclear inactivation in the two stocks can be largely accounted for in terms of the larger amount of total nucleic acid in the cytoplasm of the macroconidia. Thus the relative and absolute efficiencies support the contention that *only quanta absorbed within the nucleus can lead to the inactivation of the nucleus.*

In view of the conclusions in the last paragraph it seems that the action spectrum should be corrected for the absorption in the cytoplasm. This correction serves simply to lower the inactivation cross-sections relative to that at 265 mμ by a few per cent. However, the interesting question arises of why the absorption of some 10^{19} ultraviolet quanta per cm^3 of cytoplasm has so little effect on the conidial viability. It would seem that each cytoplasmic enzyme system is present in large numbers and/or that the association of these systems *in vivo* with solid structures such as mitochondria imparts a greater stability to them than is expected on the basis of studies on enzyme solutions (13).

DISCUSSION

The term "non-genetic" means not inheritable; the criterion for lethality is (essentially) the inability of the nucleus to multiply. Thus, an operational distinction between a genetic and non-genetic lethal effect presents some difficulties. If the symbols A, A', and A'' are used to designate a normal nucleus, that nucleus contaning a lethal mutation, and that nucleus with some non-genetic lethal effect respectively, then an operational definition

of genetic (and non-genetic) depends on the ability to demonstrate that A' and A'' multiply according to the following scheme

[14] $$A' \longrightarrow A' \qquad A'' \longrightarrow A.$$

The distinction may be made clearer by noting that the special conditions under which A' divides must be provided at every generation in order for the progeny of A' to divide, in turn. On the other hand, the conditions under which A'' will divide need be provided only once. Thus A'' requires only a sort of "pump priming." The distinction between the genetic and non-genetic lethal effects is clearly meaningful, since it is logically possible to demonstrate the sequences

[14 a] $$A' \xrightarrow{C'} A' \xrightarrow{C'} A' \xrightarrow{C'} \cdots, \quad A'' \xrightarrow{C''} A \longrightarrow A \longrightarrow \cdots,$$

where C' and C'' represent the special conditions under which A' and A'', respectively, will divide.

A glance at the scheme in [14] shows that the nucleus A' can be kept in a stock indefinitely, but the condition A'' is essentially transitory—as soon as A'' divides the evidence for its existence vanishes. Thus the existence of nuclei containing a non-genetic lethal effect must be inferred from data such as the kinetics of inactivation.

The presence of a lethal mutation can be demonstrated by showing that a conidium heterokaryotic for the suspect nucleus can grow, indicating that the nucleus is reproducing itself in the heterokaryon. On the other hand, a conidium homokaryotic for that nucleus cannot grow. This is the basis for the method used by Atwood (2) in proving that the frequency of nuclei bearing lethal mutations is much lower than is expected on the assumption that lethal mutations are responsible for the inactivation of multinucleate conidia. (More specifically, his most ingenious method consists of growing a heterokaryon and allowing it to conidiate. Normally the conidia will be of three types: heterokaryons between the two kinds of nuclei, and the two kinds of homokaryotic conidia. The failure of either type of homokaryon to grow indicates the presence of a lethal mutation in the parental nucleus.)

As an example of how to demonstrate the non-genetic lethal effect consider the following experiment: (Atwood, in press) a heterokaryon is formed between the two auxotrophs O, requiring ornithine to grow, and *L*, requiring lysine to grow. The heterokaryon can grow, of course, on minimal medium (devoid of ornithine or lysine). If this heterokaryon is irradiated, then the kinetics of inactivation show that the conidial inactivation is a con-

sequence of the nuclear inactivation. Thus, at the dose at which equation [4] begins to apply, there is only one viable nucleus per (surviving) conidium; for that reason the survival curve of the multinucleate conidia at high dose resembles that of the uninucleate conidia. Therefore, at high dose, the surviving heterokaryons should be of two types—those containing viable O nuclei and those containing only viable L nuclei. Yet on media containing ornithine and/or lysine the number of survivors is increased very little over that on minimal medium. This indicates that in the presence of an uninjured nucleus the inactivated nucleus can reproduce itself normally;—i.e., the change in the nucleus due to the radiation was not transmitted. Thus the lethal effect, by definition, is non-genetic.

The literature contains many additional demonstrations of recovery from the effects of ultraviolet radiation. The possibility of recovery is implicit, of course, in the definition of a non-genetic lethal effect. For example, Roberts and Aldous (17) have shown that the recovery of *E. coli* is markedly dependent on a host of apparently trivial factors in the pre- and post-irradiation treatment of the organisms. Then, there is this common experience in screening for auxotrophic mutants: a number of colonies will arise only upon the addition of "complete" medium to the "minimal" medium in which the organisms are inoculated; yet of these colonies very few are mutants.

In relating the non-genetic effect to a cellular structure it must be remembered that this structure shows a one-to-one correspondence with the number of nuclei and that the probability of inactivating it is large compared to the probability of inducing a lethal mutation at any given locus. (Indeed, the probability of inactivating this structure is about equal to the probability of inducing at least one lethal mutation, so that the sensitivity of this structure is at least two orders of magnitude greater than the sensitivity of any given locus.) This evidence for a single sensitive unit per nucleus immediately suggests the nucleolus. Now, the action spectrum for morphological changes in the nucleolus is a little different from that found here (Carlson and McMaster, unpublished); but the effect could involve the nucleolar organizer. It is possible, of course, that an extra-nuclear organelle, such as the centriole, is involved whose number per cell corresponds to the number of nuclei.

The close relationship between the genetic and nongenetic processes demonstrated by the action spectra and the photoreactivation data demands a more critical investigation of the assumption that these processes are independent. The first consequence of this assumption is equation [7] which can be rewritten as

[15] $$L = L_0(1 - e^{-k_2 D})$$

where L is the number of nuclei containing at least one lethal mutation in a test of L_0 nuclei. At low dose this relation becomes

[16] $$L = (L_0 k_2) D.$$

Now these are precisely the relations found between the frequency of recessive lethal mutations and the dose of *ionizing* radiation [14]; but they are at complete variance with the strange relationship of these quantities when ultraviolet radiation is used. With this non-ionizing radiation the frequency of mutations, among the surviving organisms, increases up to a certain dose and then begins to decrease again with greater dose [8]. Equation [7] can be tested in another way: combining it with equation [9] we can write

[17] $$\log S(n) = \log \bar{n} + (k_1/k_2) \log S_2.$$

This predicts that the log of the fraction of nuclei without a lethal mutation plotted against the log of the fraction of surviving (multinucleate) conidia should be a straight line of slope k_1/k_2. However, when Atwood's data (3) are plotted in this fashion they show considerable variation from the predicted straight line. It seems, thus, that the assumption of independence is untenable; but the nature of the interaction of the two processes is most puzzling since the non-genetic process acting alone leads to a simple exponential relation of survival to dose and the two processes acting together also produce an exponential relationship of survival to dose. Indeed, at this point the reality of the two processes may be questioned. It is necessary, therefore, to emphasize that when the multinucleate conidia show twenty per cent survival, among the survivors almost fifty per cent of the nuclei contain at least one lethal mutation (3). It seems unbelievable that these lethals do not contribute significantly to the inactivation of the uninucleate conidia. But even on the extreme assumption of one locus per nucleus the frequency of lethal mutations is far too low to account for the inactivation of the multinucleate conidia.

Ultimately, the biological effects of radiation must be related to chemical transformations; the action spectrum offers one of the few clues to the nature of the chemical species involved. While there are many assumptions implicit in the identification of the action spectrum with the absorption spectrum of a chemical compound (12) there can be little doubt that both the genetic and non-genetic processes are initiated by the absorption of an ultra-

violet quantum in the nucleic acid of the conidia. The reason for this assurance lies in the many action spectra, using both inactivation and mutation as criteria for the radiation effectiveness, which are very similar to those in figure 7 (11, 12); it lies, also, in the evidence that nucleic acids play a central role in the reproductive cycle of all organisms. Beyond this primary photochemical act spreads a realm of speculation whose exploration may be of considerable heuristic value, but this exploration is not germane to the primary objective of this work: the role of the nucleus in the inactivation process.

Returning to this problem it can be said that the kinetics of inactivation furnish strong support to the hypothesis that the inactivation of the conidia is a consequence of the inactivation of the nuclei; and this is in line with the general findings in radiobiology that the nuclei constitute the more sensitive portion of the cell (18). The validity of this hypothesis, in turn, furnishes strong support to the theory advanced by Atwood and Norman (3) to account for multi-hit survival curves, for this theory is the basis for the analysis of the kinetics data. Finally, it is important to emphasize that the term "process" is really an abbreviation for "the class of processes"; for there is no question that each "process" can be subdivided into several subclasses. In the case of the genetic effects this subdivision has already been carried out to a large extent (14). Similar subdivision of the class of non-genetic processes seems inevitable; for the heterogeneity of an irradiated population increases in direct proportion to the number of criteria used in detecting the effects of radiation.

SUMMARY

The inactivation of *Neurospora* conidia by ultraviolet radiation is a consequence of the inactivation of the nuclei. The nuclei exhibit a kinetics of inactivation which is first order with respect to the number of (viable) nuclei. Arguments based on the efficiency of inactivation indicate that only quanta absorbed within a nucleus can lead to its inactivation.

There are at least two sets of inactivation processes. One involves the production of lethal mutations, the other is a non-genetic (not inheritable) process. The two processes are apparently intimately connected: they yield the same action spectrum and the effects of each are reduced by photo reactivation. The assumption that the two processes are independent leads to predictions which are at variance with the observed dependence of the frequency of lethal mutations on dose of ultraviolet radiation.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge my indebtedness to Professor F. J. Ryan for his encouragement and help and to Professor K. C. Atwood for his many stimulating criticisms. I thank Professors L. C. Barth, R. M. Noyes, and A. W. Pollister and Dr. T. Hsia for the loan of various items of equipment; Miss Rachel McMaster for help with the cytological work; Professor T. Hayashi for generosity with laboratory space and equipment, and Dr. A. Hollaender for the opportunity to inspect his ultraviolet set-up.

This work was made possible by an AEC Predoctoral Fellowship in the Biological Sciences administered by the National Research Council.

REFERENCES

1. Atwood, K. C., *Biol. Bull.*, **99**, 332 (1950).
2. Atwood, K. C., *Genetics*, **35**, 95 (1950).
3. Atwood, K. C., and Norman, A., *Proc. Natl. Acad. Sci.*, **35**, 696 (1949).
4. Barrat, R. W., and Garnjobst, L., *Genetics*, **34**, 351 (1949).
5. Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678 (1945).
6. Caspersson, T., Cell Growth and Cell Function. W. W. Norton, New York, 1950.
7. Haxo, F., *Arch. Biochem.*, **20**, 400 (1949).
8. Hollaender, A., Sansome, E. R., Zimmer, E., and Emmons, C. W., *Am. J. Bot.*, **32**, 226 (1945).
9. Kelner, A., *J. Bact.*, **57**, 363 (1949).
10. Latarjet, R., and Ephrussi, B., *Compt. rend.*, **229**, 306 (1949).
11. Lea, D. E., Actions of Radiations on Living Cells. The Macmillan Co., New York, 1947.
12. Loofbourow, J. R., *Growth Symposium*, **XII**, 75 (1948).
13. McLaren, A. D., *Advances in Enzymol.*, **9**, 75 (1949).
14. Muller, H. J., *J. Cellular Comp. Physiol.*, **35**, 9 (1950).
15. Novick, A., and Szilard, L., *Proc. Natl. Acad. Sci.*, **35**, 591 (1949).
16. Noyes, W. A., and Leighton, P. A., The Photochemistry of Gases. Reinhold, New York, 1941.
17. Roberts, R. B., and Aldous, E., *J. Bact.*, **57**, 363 (1949).
18. Whiting, A. R., *Proc. Natl. Acad. Sci.*, **36**, 368 (1950).

THE AMINO ACID METABOLISM OF THE DEVELOPING SEA URCHIN EGG

TRYGGVE GUSTAFSON and MAJ-BRITT HJELTE

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Received March 5, 1951

The sea urchin egg, during earlier development, is morphogenetically a very labile system. For example the addition of lithiumions (Li) to the sea water in the earlier stages changes its morphogenesis profoundly, the entire egg sometimes developing into an entoderm (23, 42, 15). If on the other hand, the eggs are treated with thicoyanate ions in calcium-free sea water before fertilization a reverse effect may be shown with the whole egg developing into an ectoderm-like vesicle (42).

One may suppose that these ions interfere with the processes of synthesis upon which histogenesis is based. To get further insight into the mode of action of these ions a study of the content of various amino acids in the development of both normal and Li-treated eggs was carried out.

MATERIALS AND METHODS

Sea urchin eggs, *Paracentrotus lividus*, were collected at Station Biologique de Roscoff, Bretagne, France. 99 to 100 per cent of the eggs could be fertilized. The eggs were cultivated in dilute suspensions in glass trays (40). The temperature was kept constant at 22° C and the stages taken for analysis were always chosen with reference to the respiration curve given by Lindahl (41).

It is known (30) that a long Li treatment at a low concentration has the same morphogenetic effect as a shorter treatment with a higher concentration. For practical reasons longer treatment with a comparatively weak Li concentration was chosen, using 95 parts sea water of salinity 3.5 per cent and 5 parts isotonic Li solution, i.e. a final Li concentration of 0.028 Mol. The treatment was always started immediately after fertilization and was interrupted at the fixation after 4, 7, 12, or 18 hours. If the larvae were cultivated for a longer time they were always washed repeatedly after 20 hours and transferred into normal water. A 12 hour treatment under these conditions gave rise to strongly vegetalized larvae with only a small ectoderm vesicle. The eggs were washed and centrifuged down at various stages and were frozen on a galvanized plate, previously chilled to —20° C. The material was then dried in the frozen state and stored in a vacuum desiccator over anhydrous silica gel.

For the analysis, equal parts of three different preparations at the same stage were

mixed and 250 mg were hydrolyzed in a closed pyrex glass ampoule with 3.5 ml 10 per cent HCl for 10 hours at 120° C. This treatment seems to be sufficient in most cases. For the analyses of tryptophane the hydrolyses were performed with NaOH which had been previously autoclaved with cysteine to protect the tryptophane from oxidation during the hydrolysis (37).

The first analyses were performed, using absorption methods—activated charcoal and ion exchange technique. Colorimetric methods were also tried. However none of these methods seemed very suitable for the analysis of numerous samples containing minute amounts of amino acids. Microbiological assay technique was then tried which was more convenient and gave more accurate results.

The microbiological methods were basically those of Stokes *et al.* (56) and of Henderson *et al.* (22) cf. tab. I. Estimation of the bacterial growth was always made by means of titration, generally electrometrically. The analyses were made at 3 or 4 different concentrations, each concentration in triplicate. Several separate determinations were made for most of the amino acids and generally two different hydrolyzates were examined. The different results for the same amino acid generally showed excellent agreement. Sometimes, however, the magnitude was somewhat different in different series but the form of the curve was always the same. The recorded curves are generally based on the mean of several series of determinations. The amino acid nitrogen is expressed as per cent of the total (Kjeldahl) nitrogen of the egg. The nitrogen of the egg is constant at least up to 32 hours of normal development. After this time no further measurements were performed. (Cf. Horowitz' data (28) for the *Urechis* egg and his critizism of earlier literature data (12, 21) on the nitrogen content of developing sea urchin eggs.) Thus the changes in the different amino acids, up to this time, are not distorted by changes of the total nitrogen of the egg, at least not before 32 hours.

Paper chromatographic methods were used in the study of the free amino acids. Two-dimensional analyses were performed on desalted 75 per cent alcohol extracts on Munktell OB filter paper with collidine and phenol. Non protein nitrogen for Kjeldahl and Van Slyke amino nitrogen determinations was extracted from the eggs with tungstic acid.

The glutamine determinations were performed after hydrolysis of whole dry egg samples by steaming them in an autoclave at 100° C in a neutral 0.33 molar phosphate buffer for 45 minutes. This method is an adaptation of that developed by Hamilton (20). It has been carefully checked on pure glutamine, egg extracts and the extracted egg residue. The Conway technique (10) and nesslerization were used for the ammonia determinations.

The difficulty of complete Kjeldahl combustions of sea urchin eggs has been emphasized by Örström (62). He also gives striking examples of the great variability between the nitrogen values given by different authours for the same amount of *Paracentrotus* eggs. We have thus paid much attention to the combustion technique. Since the superiority of Hg-containing media has been repeatedly stressed (43, 45) the medium of Arnold-Wedemeyer (1) was chosen. Complete combustion was secured by the use of persulphate (59). Our figures on total non-protein nitrogen and the relationship between total N and P deviate from those reported or calculated from Örström's papers (61, 62). These deviations indicate incomplete whole egg combustions even in Örström's analyses.

Results obtained with different methods for the same amino acid always agree very well. In the following table some microbiological results from *Paracentrotus lividus* are compared with results obtained by means of other methods from a related species, *Strongylocentrotus droebachiensis.*

Tryptophane	Microbiologically	0.67 per cent
	Colorimetrically (27)	0.65 per cent
Arginine	Microbiologically	10.68 per cent
	Colorimetrically (8)	10.96 per cent
Acid amino acids	Microbiologically	11.40 per cent
	Ion exchange technique (56)	10–12 per cent
Basic amino acids	Microbiologically	20.26 per cent
	Ion exchange technique (56)	22 per cent

The reliability of the microbiological methods is also supported by the Van Slyke determinations on acid hydrolysates. These determinations were performed with iodine in the reaction mixture (33) in order to get theoretical values for glycine and cysteine. At 23° C and 3–4 minutes reaction time too high values seem to be obtained only with lysine (14, 33, 52). Some nitrogen must however also be evolved from amino purines etc. As seen from the curve (fig. 1) there is an excellent agreement between the Van Slyke determinations and the values obtained after summation of the microbiological amino nitrogen values from acid hydrolysates.

ABBREVIATIONS TO BE USED BELOW

Nitrogen: N; Total Kjeldahl N: Total N; Total non-protein N extractable with tungstic acid and determined by Kjeldahl combustion: NPN; Van Slyke amino N extractable with tungstic acid: Van Slyke N.

RESULTS

The main results may be best described by the accompanying tables and curves, most of which are based on the values of table I in a preliminary paper (18). The present values have however been revised after further assay and by omission of some of the earliest determinations where colorimetric titrations were used. The differences between the preliminary and the revised values are as a rule low. After revision, however, also very small changes in the amino acid composition are discernible although the significance of the smallest changes may be uncertain.

In unfertilized eggs 88.9 per cent of the Total N can be accounted for as amino acid N, see fig. 2. The rest according to calculations based upon determinations from this and other species (58, 60, 61) roughly corresponds to nucleic acid, phosphatide, and protein amide N. In later normal stages

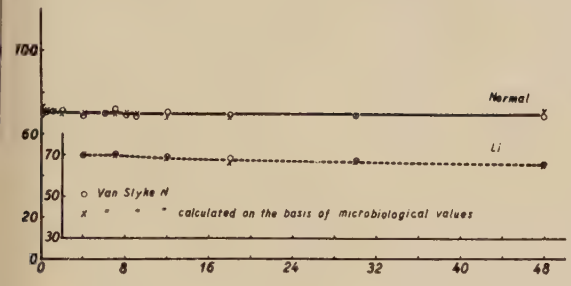


Fig. 1.

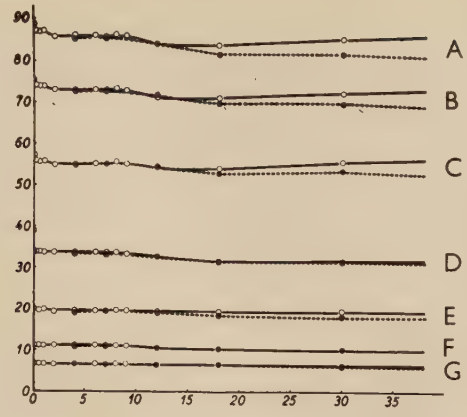


Fig. 2.

Fig. 1. Comparisons between the amino N values from Van Slyke determinations and the sum of the microbiological α-amino N values from acid hydrolysates.

Fig. 2. Quantitative developmental changes in: A) Amino acid N. B) Amino acid amino N. C) Neutral amino acid N. D) Neutral, non-glycine amino acid N. E) Basic amino acid N. F) Acid amino acid N. G) Basic amino acid amino N. The values of this curves are based upon the summation of microbiological determinations of individual amino acids. The curves give the N content of each group in per cent of Total N of the respective stage. Unbroken curves: normal stages, dotted curves: Li-treated stages.

the per cent of Total N is somewhat lower. In 18 H stage only 83.8 per cent of Total N can be accounted for as amino acid N. In Li-treated material the values are, as a rule, lower than in normal development and shows approximately a steady drop from 7 to 48 H after fertilization. No attempt has been made to estimate the amounts of hydroxy-amino acids, betaines, protein amide, and amines etc. which may account for the drop in total amino acid N values.

Glycine is the amino acid which dominates with 23.10 per cent N in the unfertilized eggs while tryptophane is present in very small amounts, 0.67 per cent in the same stage. The molecular ratio between glycine and tryptophane is thus about 69:1, see table I.

As will be seen from the curves, Fig. 2, the amount of total, basic, neutral, and acid amino acid N is remarkably constant during development. Certain small changes in connection with gastrulation may, however, be discernible. Li causes minor deviations from the normal curves.

If for each stage the per cent change from the unfertilized egg values is plotted against time the individual amino acids show a more differentiated

TABLE I

A, B) Amino acid composition of the unfertilized *Paracentrotus* egg. C, D) Organisms and basal medium used for the microbiological assay (reference number). E) Number of complete assay series with different methods.—The amino acid composition is expressed, A) as per cent amino acid N of total egg N (amino acids listed after decreasing values), B) as molecular ratio between the different amino acids and the one present in smallest amount.

Amino acid	A % N of Total N	B Mol ratio	C	D	E
Glycine	23.10	69.0	Leuconostoc mesenteroides	(22)	3
Arginine	10.68	8.0	»	(22)	6
Lysine	6.58	9.8	»	(22)	3
			Streptococcus	(55)[1]	1
Aspartic acid	5.93	17.7	Leuconostoc mesenteroides	(22)	6
Valine	5.72	17.1	Streptococcus faecalis	(55)	1
			Lactobacillus arabinosus	(22)	2
Glutamic acid	5.47	16.3	»	(22)	6
Serine	4.74	14.2	Leuconostoc mesenteroides	(55)	3
Leucine	4.61	13.8	Streptococcus faecalis	(55)	1
			»	(22)	1
Isoleucine	3.78	11.3	»	(55)	1
			Leuconostoc mesenteroides	(22)	2
Alanine	3.53	10.5	Streptococcus faecalis	(22)[2]	2
Threonine	3.12	9.3	»	(55)	1
			»	(22)	1
Histidine	3.0	3.0	»	(55)[1]	1
			»	(22)	1
Proline	2.51	7.5	Leuconostoc mesenteroides	(2)	1
			»	(22)	2
Phenylalanine	2.00	6.0	Lactobacillus delbrücki	(22)	3
Methionine	1.40	4.2	Streptococcus faecalis	(55)[1]	1
Tyrosine	1.26	3.8	Lactobacillus delbrücki	(55)	1
			Leuconostoc mesenteroides	(22)	1
Cystine	0.77	2.3	»	(48)[3]	3
Tryptophane	0.67	1.0	Streptococcus faecalis	(37)[4]	2

picture, Fig. 3, e.g. whereas threonine shows a high degree of constancy glycine and some other amino acids show rather striking changes.

Besides a small but definite drop in the content of a few amino acids

[1] Succinate-buffer substituted for acetate. — Xanthine added for the methionine assay.
[2] Acetate, Mn and Mg according to (55), no citrate.
[3] Pepton-medium according to (48), vitamines according to (22).
[4] Lipids extracted from the samples before hydrolysis, alkaline hydrolysis.

during the first 1–2 hours of development, the period of determination generally is remarkably constant in the egg amino acid composition.

In the mesenchyme-blastula or during gastrulation most of the amino acids show a certain drop, others remain constant as threonine while still others show an increase as glycine. Also alanine shows an increase preceeded by the usual drop at gastrulation. In still other cases, e.g. lysine and histidine, the scatter makes conclusions about smaller details impossible. The changes generally do not exceed 10 per cent of the amounts in the unfertilized egg.

Li treatment does not essentially change the curves of certain individual amino acids, while in other cases, e.g. glycine, glutamic acid, serine, alanine, lysine and arginine the treatment has clearly changed the curves. In still other cases the changes are less marked but there seems to be significant differences in the same direction as in more clear cases.

The NPN has a remarkable high value—27 per cent of the total N in the unfertilized egg. This value is fairly constant during development, especially in Li-treated eggs, see Fig. 4.

In the unfertilized egg 21 per cent of the Total N, i.e. 78 per cent of the NPN, consists of Van Slyke N. Besides minor fluctuations in the blastula there is a definite drop to 17.6 per cent during gastrulation and a rise in the pluteus to about the original value. After Li treatment these changes in the NPN fraction are much weaker.

The difference between NPN and Van Slyke N values corresponds to a fraction which contains peptides etc. The N values for this fraction changes between about 3 and 8 per cent of Total N, see fig. 5. The most important changes in this fraction are a strong reduction in the mesenchyme blastula and a dramatic rise during gastrulation. Li treatment causes a shift of the normal minimum to an older stage and reduces the amplitude markedly.

From paperchromatographic analyses the NPN for the most part consists of glycine, but most of the other common amino acids can be shown. The picture is essentially the same with extracts from normal or Li-treated stages. Certain important changes have, however, been noted. Glutamine has been demonstrated in extracts of earlier stages. In advanced blastula, glutamine begins to disappear, but in Li-treated material this disappearance is comparatively less accentuated.

Quantitative determinations of free glutamine are in good agreement with the semi-quantitative paperchromatographic determinations, Fig. 6. They support Örström's (61) statement about a rapid accumulation of free glutamine a few minutes after fertilization. After an early peak in glutamine

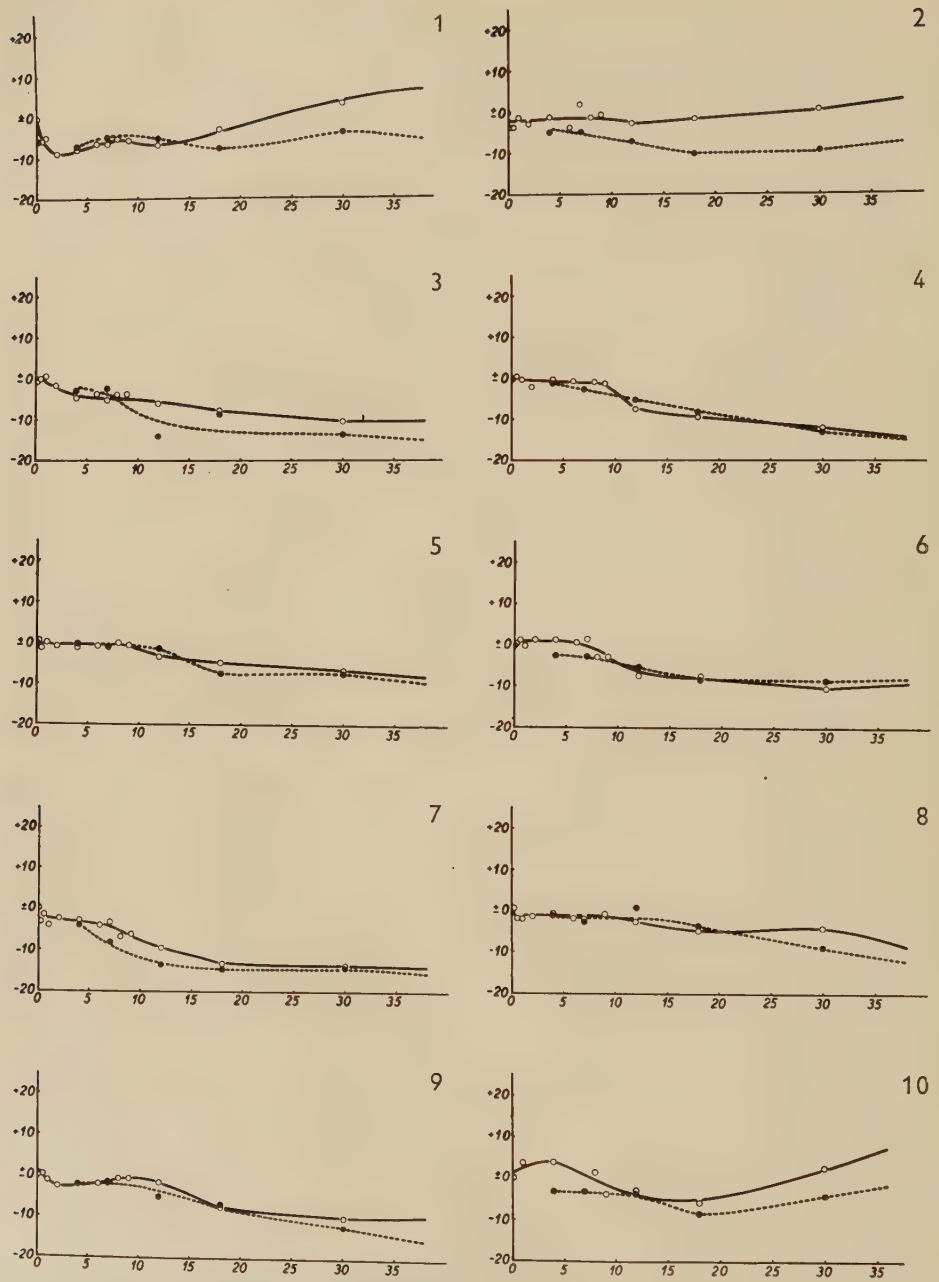
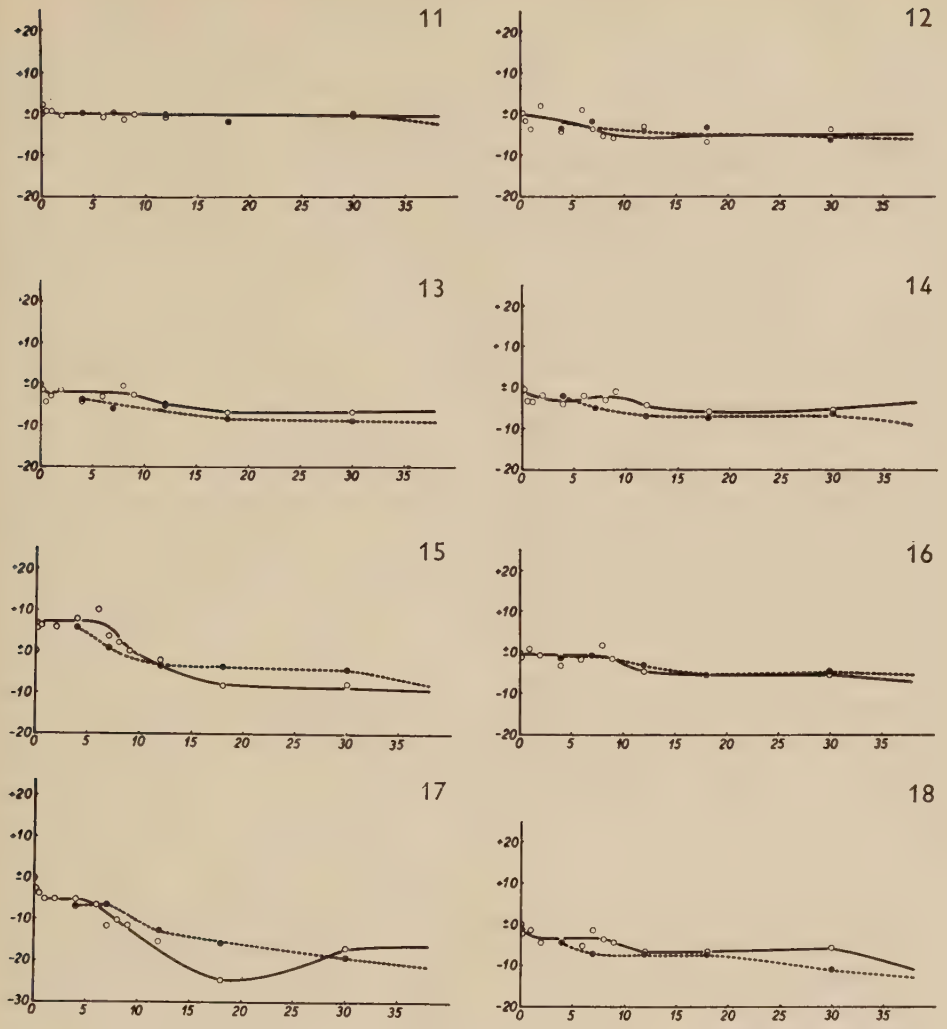


Fig. 3. Developmental changes in the amino acid concentration expressed as the per cent difference between the unfertilized egg values and those of respective developmental stage. 1)



Glycine, 2) arginine, 3) lysine, 4) aspartic acid, 5) valine, 6) glutamic acid, 7) serine, 8) leucine, 9) isoleucine, 10) alanine, 11) threonine, 12) histidine, 13) proline, 14) phenylalanine, 15) methionine, 16) tyrosine, 17) cystine, 18) tryptophane.
Unbroken curves: normal stages, dotted curves: Li-treated stages.

content, which has however not been found in every series of egg material, there is a steady accumulation in glutamine with a maximum in the blastula. After that there is a rapid drop in the glutamine content. In older stages glutamine begins to increase again. After Li treatment both accumulation and the reduction of the glutamine level is checked.

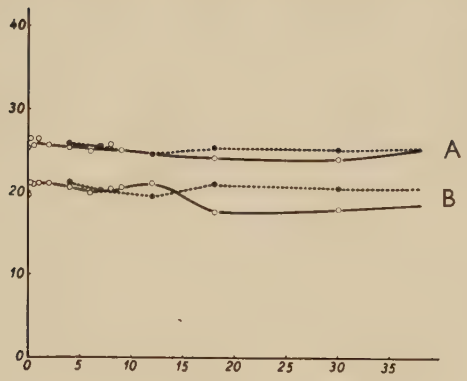


Fig. 4.

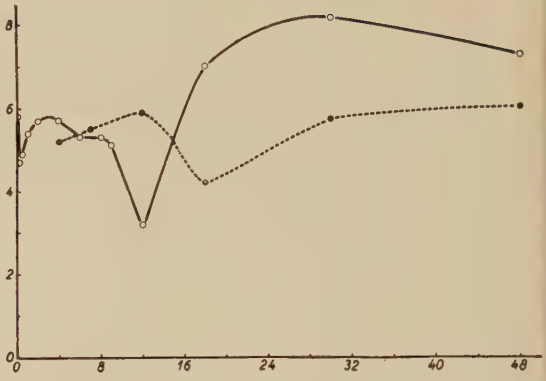


Fig. 5.

Fig. 4. Quantitative developmental changes in: A) Non-protein N (NPN). B) Non-protein amino N (Van Slyke N). The values are expressed as per cent N of the total N of respective stage. Unbroken curves: normal stages, dotted curves: Li-treated stages.

Fig. 5. Quantitative developmental changes of the "peptide-containing fraction" (non-protein non-amino N), expressed as per cent N of total N of respective stage. Unbroken curve: normal stages, dotted curves: Li-treated stages.

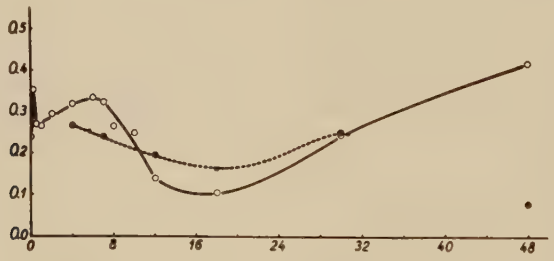


Fig. 6. Quantitative developmental changes in the concentration of glutamine, expressed as per cent amide N of Total N of respective stage. Unbroken curve: normal stages. Dotted curves: Li-treated stages.

It has been possible to demonstrate the presence of peptides in the alcohol extracts by means of paper chromatography. According to preliminary studies peptides arise at gastrulation but this observation needs further support.

Örström (62) has studied the changes in NPN and Van Slyke N occurring at fertilization of the *Paracentrotus* egg. His absolute values are much higher than ours. According to both investigators, however, there is an increase in both fractions between fertilization and the 10 minutes stage. This is at least evident from Örström's curves.

DISCUSSION

The main purpose of this work has been to characterize the different periods of egg development by studying the changes in amino acid composition. Since Li treatment profoundly interferes with the development by checking the ectoderm formation it has also been the purpose to see how Li interferes with the amino acid level. By comparing the Li curves with the normal ones we have tried to get some information about the localization of the changes to the animal and vegetal regions of the egg or more correctly to the animal and vegetal direction of development.

The recorded changes are with a few exceptions rather small and may escape the efforts of investigators using less sensitive quantitative methods. Even if these changes are small they may be important and could be expected to be much greater if more or less inert material such as yolk could be removed from the eggs before fertilization or if eggs from a species with very little yolk could be studied. Thus we cannot agree with the statement (26), that "if there is to be an embryonic chemodifferentiation within the cellular protein components it apparently does not occur at the level of the amino acids . . ."

Apart from certain changes immediately following fertilization the first 7–8 hours of development show only slight variations in amino acid composition of the egg. This seems true for normal development. Changes do not generally occur until mesenchyme blastula or early gastrula stage. This may indicate that there is a relatively weak protein or at least amino acid metabolism during the period of determination. This is intensified at the onset of visible differentiation.

The same conclusion as above can be drawn from enzymic and other studies on developing sea urchin eggs. Thus it has been demonstrated that the eggs have a constant and relatively weak alkaline phosphatase activity up to onset of gastrulation. After this time there is a large and rapid rise (16, 44). Studies on the activity of apyrase, glutaminase and succinodehydrogenase demonstrate the same kind of changes in activity during development (17).

Serological studies (47) give further support to the above conclusion regarding great changes in protein metabolism at gastrulation since new antigens make their first appearance only after the onset of gastrulation. Furthermore the ammonia production is weak if derived from pregastrula stages. Homogenates from gastrulating and later stages produce increasing amounts

of ammonia which are roughly proportional to the age of the stage (17). The intensification of the N metabolism when mesenchyme blastula or gastrula stage has been reached is also evident from the quantitative studies on glutamine, Van Slyke N, the peptide containing fraction and paper chromatographic work.

Experimental work on intact eggs also support the conclusion above. So the egg first requires sulphate in late blastula or at onset of gastrulation (24, 25, 40). Eggs after disperm fertilization and merogons of distantly related echinoderms first begin to show signs of disorder at about the same period (5, 51). Dorsoventral development of *Psammechinus* eggs may be checked by cultivation in extremely dilute detergent solutions if the treatment begins before mesenchyme blastula stage (19). The intensity of respiration, after being constant for some hours, begins to rise again at about the same stage (41).

Cytological evidences stress that the nuclear activity is intensified around gastrulation as shown by the increase in Feulgen staining (7) and nuclear alkaline phosphatase activity (36). Also the nucleolar apparatus is better developed at gastrulation than in the earlier stages according to Monné (unpublished).

Thus important processes, e.g. of synthetic nature, probably controlled by an increased nuclear activity, starts simultaneously with the onset of visible differentation, i.e. in mesenchyme blastula and gastrula stages.

The preceeding period of determination seems, at least with regard to protein metabolism, to be mainly characterized by small scale processes, paralleled by a low nuclear activity (15). As shown from experiments with isotopic N (29) ammonia can be incorporated into the proteins of *Paracentrotus* eggs in an increasing velocity even during the cleavage and early blastula stages. This incorporation may reflect a small scale process of great importance for determination (15).

In a few cases there occurs, after an earlier drop, a resynthesis of amino acids during differentiation, i.e. of glycine and alanine. This synthesis, which probably consumes ammonia, liberated at the breakdown of other amino acids, does not occur in vegetalized material, at least not to the same extent as in normal larvae. This is in good agreement with enzymic studies (17) which show that the rise in activity of apyrase and glutaminase in early gastrula is very much checked by a previous Li-treatment. The suppression of the ectodermal development is evidently paralleled by a checked synthesis on both the amino acid and enzymic levels. Thus, whereas the animal development seems to be characterized by important synthesis pro-

cesses the vegetal synthesis seems to be much weaker. The same conclusion could be drawn from the discussion below concerning glutamine and the peptide containing fraction of NPN.

It has been shown above that the amount of a series of amino acids begins to drop earlier in Li-treated material than in normal development. This effect is quite clear in e.g. glutamic acid, alanine, serine, arginine, and lysine while in a number of cases it is very slight and the significance could be doubtful. In conformity with the discussion above this may indicate that the protein metabolism begins earlier in vegetal material than in animal parts of the egg even if the intensity during further development is weak. This suggestion is partly supported by experimental methods indicating that determination is earlier finished in vegetal material (30, 40, 50). Since the entoderm normally constitutes a relatively small portion of whole larva these earlier changes are normally more or less masked.

The staining properties of the yolk is very much changed at gastrulation (46) which may indicate a beginning breakdown of this material. According to Monné (46) the yolk has the staining properties of a highly basic substance. One could thus assume that it is rich in arginine and lysine. These amino acids should thus be transferred from the desintegrating yolk granulae for building up other structures rich in basic proteins. Combustion or at least conversion into other compounds mainly takes place after Li treatment. It may thus be characteristic for the vegetal direction of development.

Lindahl (40) demonstrated the presence of a phenolsulphatase in the sea urchin egg. He suggested that sulphate is responsible for the detoxification of the aromatic waste products from a protein combustion. This should begin at about gastrulation and be associated with the vegetal development. Our results support the hypothesis of Lindahl since they indicate a parallel between the onset of gastrulation and the drop in the content of various amino acids, probably including the aromatic ones. Furthermore, as mentioned above, after Li treatment this drop is intensified in a number of cases, sometimes beginning already in the blastula.

The drop in sulfur amino acids at gastrulation or even before would be sufficient for detoxification of all the aromatic waste products, assuming that all the sulfur amino acids are combusted to sulphate. At least a part of them may, however, be transformed into other sulfur compounds. Taurine may be a possible example since it is a very common metabolite in marine invertebrates, even in echinoderms (3, 26, 34, 38).

Glutamine accumulates during a period of development where protein

synthesis evidently is low while the glutamine content decreases when protein synthesis accelerates. An analogous result from the yeast *Turolopsis* has been reported by Roine (49), who concludes that glutamine is consumed for protein synthesis.

There is a rise in glutaminase activity at gastrulation (17) while the glutamine level does not rise until very advanced stages. This may indicate that the turnover rate of glutamine for protein synthesis is very much increased from the time of gastrulation. Thus protein synthesis may not only be dependant upon glutamine accumulated during earlier cleavage stages. After Li treatment the slower rise in glutaminase activity and the higher level of glutamine may indicate a slower rise in glutamine turnover rate. This should indicate a lower protein synthesis after Li treatment. This conclusion is also suggested by other kinds of evidences.

The synthesis of glutamine is dependant upon ATP-supply (11, 6, 53) and thus upon respiration and glycolysis (35). One could thus assume that the lower accumulation of glutamine in Li-treated cleavage stages is the result of Li's depressing effect on respiration. The Li effect on glutamic acid content of the egg, cf. above, may also be important in this connection.

The glutamine might be especially important for the animal development is also suggested from the observations that glutamine added to the sea water fortifies the development of the ciliary tuft of the animal sea urchin egg fragments (31, 32).

The difference between the NPN and the Van Slyke N values must correspond to a fraction that among other things contains peptides. If proteins are synthesized from peptides one could expect that this fraction should show changes during development depending upon changes in the relative rate of peptide synthesis and peptide consumption for protein synthesis.

Apart from changes immediately after fertilization the curves suggest that there is a peptide accumulation during early cleavage stages when protein synthesis from various kinds of evidences seems to be weak.

During intermediate cleavage stages the consumption seems to dominate over peptide synthesis and especially at mesenchyme blastula stage there is a strong depletion of accumulated peptides corresponding to an intensified protein synthesis. This early part of the curve thus is similar to the glutamine curve.

When gastrulation begins there seems to be an intensive peptide synthesis parallel to a very much intensified enzyme synthesis and an increased glutamine turnover rate.

The reduced depletion of peptides in Li-treated mesenchyme blastula is

in good agreement with other evidences for reduced protein synthesis in this material. However one cannot exclude the possibility that the Li curve is also affected to a certain degree by a certain unspecific retardation of development. The reduced accumulation in later developmental stages seems to be correlated with a slower protein synthesis and a lower glutamine turnover rate.

One could possibly suggest that there is a casual connection between the glutamine consumption and the formation of peptides. The energetic similarity between the amide and true peptide linkages (53) should be kept in mind in this connection.

An elevated concentration of free amino acids has been recorded for regenerating liver and for fetal tissues of mammals by Christensen *et al.* (9) who have also discussed the association between this phenomenon and rapid growth. Horowitz (28) found that the amount of NPN is much higher in the egg and larvae of *Urechis* than in tissues of the adult. He speculated on the inability of the embryo to synthesize more of its amino N into proteins. He supposed that it would be an advantage for organisms, which must undergo a certain degree of metamorphosis, in keeping its N in a mobile state. In this way it could avoid a more important histolysis such as is found in insects. He also suggested that there could be a deficiency in one or more of the essential amino acids which must be supplied from food before synthesis can occur. However it cannot be stated as yet that the amount of free amino acids other than glycine is especially high in the *Paracentrotus* egg. The idea that certain amino acids must be supplied before synthesis of proteins can proceed has, however, been the central theme for the work of Hörstadius and Gustafson (31) in their experiments on the influence of amino acids on development of animal egg fragments. They could demonstrate that after addition of certain amino acids to the sea water the differentiation of the second-day larva is often richer than in the control fragments in ordinary sea water.

The very high content of NPN in the eggs of *Paracentrotus* has been observed before by Örström (62). He claims that the main fraction should be glycine since this compound is present in great amounts in extracts from testicles of the sea urchin (34) and in various other marine animal tissues (39). Örström's supposition has now been confirmed by paper-chromatographic analysis.

The main fraction of the free amino acids, preferably glycine, must be an osmotic one (3, 34, 62). Örström (62) calculated the freezing point depression of extractable amino acids in the unfertilized egg. Assuming that

all the extractable amino acids behaved as glycine the value should be 1.°49 C. According to Bialaszewicz (3, 62) the inorganic electrolytes of the intermicellar fluid gives a freezing point depression of 0.°91 C. The sum of these two components, 2.°40 C, is higher than the freezing point depression of the sea water of Roscoff which is 1.°91 C. This discrepancy could indicate according to Örström (62) that some of the extractable amino acids are probably not in the free state but are bound to the proteins in some way. Örström's values on the Van Slyke N of the egg is, however, much higher than ours. (Örström 24.3 per cent, present work 19.6 per cent.) If the freezing point depression is calculated on the basis of our values the value is 1.°98 C. This agrees fairly well with that of the sea water. There is thus no reason to assume that any part of the extractable amino acids is not actually in the free state.

Summarizing, a synthesis of various kinds of observations could possibly be combined to the following picture: The protein changes which may reflect the processes of determination are weak. The period of determination thus seems to be mainly characterized by small scale processes. However, during this period also important intermediate products for protein synthesis such as glutamine and peptides are accumulated. A certain utilization of these accumulated products during the period of determination is not excluded; in fact the results indicate that this occurs. A more intensive protein synthesis first begins around mesenchyme blastula stage. Since the enzymic pattern is still deficient in certain respects at this stage a strong depletion of the glutamine and peptide reserve occurs. At onset of invagination of the intestine the glutamine turnover rate is increased paralleled by an increased peptide and protein synthesis. After Li treatment the slower rise in glutamine turnover rate is paralleled by a lower peptide and protein synthesis. The vegetal differentiation may begin earlier than the animal one. It also seems to be characterized by a weaker protein synthesis but possibly by a more intensive protein combustion than the animal development.

It may, however, be dangerous to overemphasize the equivalence between the physiological processes in a Li-vegetalized whole larva and those processes which are characteristic for the normal vegetal development. The present study may give valuable contribution to the study of isolated animal and vegetal fragments, although the ultramicromethods for amino acid and enzyme determinations are not yet very much developed. Studies of this kind may also suggest mechanisms in the complicated processes of peptide and protein synthesis.

SUMMARY

The amino acid composition of the sea urchin egg in different developmental stages has been studied. In early development the changes in amino acid composition are relatively small. Clear changes in the content of most amino acids begin to occur in mesenchyme blastula stage or during gastrulation. Glutamine and the peptide containing fraction of tungstic acid extracts show greater quantitative changes than any of the other amino acids or fractions studied. Li treatment, which checks ectoderm-formation and favours entoderm-development also interferes on the amino acid level. The present results have been discussed in relation to other results from experimental, cytological and enzymic studies. An attempt to synthesize all these findings has been made.

This work has been carried out with the aid of the Swedish Research Council, Liljewalch Jr.'s resestipendiefond, and Gustaf and Anna Retzius' fond för vetenskaplig forskning.

We wish to express our cordial thanks to Professor John Runnström for his help and interest in our work. We are also greatly indebted to the Director of Station Biologique de Roscoff, Professor G. Teissier, and to the Assistant Director, Professor P. Drach for facilitating our work in every way. We also express our cordial thanks to Miss Ingrid Hasselberg, who assisted at the collection of the material and performed a number of analyses, and to Mr. Johannes Norman for technical assistance.

REFERENCES

1. Arnold, C., and Wedemeyer, K., *Z. analyt. Chem.*, **31**, 525 (1892).
2. Barton-Wright, E. C., Emery, W. B., and Robinson, F. A., *Nature*, **157**, 628 (1946).
3. Bialaszewicz, K., *Protoplasma*, **6**, 1, (1929).
4. Boveri, Th., *Z. Naturwiss.*, N. F., **43**, 1 (1907).
5. —— *Arch. Entwicklungsmech. Organ.*, **44**, 417 (1918).
6. Bujard, E., and Leuthardt, F., *Helv. Physiol. et Pharmocol. Acta*, **5**, C 39 (1947).
7. Brachet, J., Embryologie chimique, Paris and Liège, 1944.
8. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 359 (1942).
9. Christensen, H. N., and Streicher, J. A., *J. Biol. Chem.*, **175**, 95, 101 (1948).
10. Conway, E. J., Microdiffusion Analysis and Volumetric Error, Crosby Lockwood & Ltd., London, 1947.
11. Elliot, W. H., *Nature*, **161**, 128 (1948).
12. Ephrussi, B., and Rapkine, L., *Ann. Physiol. Physico-chim. biol.*, **4**, 386 (1928).
13. Gilbert, J. B., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **180**, 209 (1949).
14. Grassmann, W., and Stadler, P., Die Methoden der Fermentforschung, Edited by Bamann, E., and Myrbäck, K., Georg Thieme Verlag, Leipzig, p. 1107, 1941.
15. Gustafson, T., *Rev. Suisse zool.*, **57**, 77 (1950).
16. Gustafson, T., and Hasselberg, I., *Exptl. Cell. Res.*, **1**, 371 (1950).
17. —— *Exptl. Cell. Res.*, **4** (1951).
18. Gustafson, T., and Hjelte, M.-B., *Arkiv Kemi*, **2**, 363 (1950).
19. Gustafson, T., and Sävhagen, R., *Arkiv Zool.*, **42** A, No. 10 (1949).
20. Hamilton, P. B., *J. Biol. Chem.*, **158**, 375 (1945).

21. Hayes, F. R., *Carnegie Inst. Wash. Pub.*, **435**, 181 (1933).
22. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
23. Herbst, C., *Z. Wiss. Zool.*, **55**, 446 (1892).
24. —— *Arch. Entwicklungsmech. Organ.*, **5**, 649 (1897).
25. —— *Arch. Entwicklungsmech. Organ.*, **17**, 306 (1904).
26. Holtfreter, J., Koszalka, T. R., and Miller, Leon L., *Exptl. Cell. Res.*, **1**, 453 (1950).
27. Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, **157**, 153 (1945).
28. Horowitz, N. H., *J. Cellular Comp. Physiol.*, **14**, 189 (1939).
29. Hultin, T., *Exptl. Cell. Res.*, **1**, 599 (1950).
30. Hörstadius, S., *Arch. Entwicklungsmech. Organ.*, **135**, 1 (1936).
31. Hörstadius, S., and Gustafson, T., Unpublished.
32. Hörstadius, S., and Strömberg, S., *Arch. Entwicklungsmech. Organ.*, **140**, 409 (1940).
33. Kendrick, A. B., and Hanke, M. E., *J. Biol. Chem.*, **132**, 739 (1940).
34. Kossel, A., and Edlbacher, S., *Z. physiol. Chem.*, **94**, 264 (1915).
35. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
36. Krugelis, E. J., *Biol. Bull.*, **93**, 209 (1947).
37. Kuiken, K. A., Lyman, C. M., and Hale, F., *J. Biol. Chem.*, **171**, 551 (1947).
38. Kutscher, F., and Ackermann, D., *Ann. Rev. Biochem.*, **2**, 373 (1933).
39. —— *Ann. Rev. Biochem.*, **5**, 460 (1936).
40. Lindahl, P.-E., *Acta Zool.*, **17**, 179 (1936).
41. —— *Z. vergleich. Physiol.*, **27**, 233, (1939).
42. —— *Quart. Rev. Biol.*, **17**, (1942).
43. Milbauer, J., *Z. analyt. Chem.*, **111**, 397 (1937–38).
44. Mazia, D., Blumenthal, G., and Benson, E., *Biol. Bull.*, **95**, 250 (1948).
45. Miller, L., and Houghton, J. A., *J. Biol. Chem.*, **159**, 373 (1945).
46. Monné, L., *Arkiv Zool.*, **1**, 101 (1949).
47. Perlmann, P., and Gustafson, T., *Experientia*, **4**, 481 (1948).
48. Riesen, W. H., Spengler, H. H., Robblee, A. R., Hankes, L. V., and Elvehjem, C. A., *J. Biol. Chem.*, **171**, 731 (1947).
49. Roine, P., *Ann. Acad. Sci. Fennicae*, Ser. A. II. Chem., **26**, 1 (1947).
50. Runnström, J., *Acta Zool.*, **9** (1928).
51. Schleip, W., Die Determination der Primitiventwicklung, Akademische Verlagsgesellschaft, M. B. H. Leipzig, 1929.
52. Schmidt, C. L. A., *J. Biol. Chem.*, **82**, 587 (1929).
53. Speck, J. F., *J. Biol. Chem.*, **168**, 403 (1947).
54. —— *J. Biol. Chem.*, **177**, 533 (1949).
55. —— **179**, 1387, 1405 (1949).
56. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
57. Tiselius, A., Drake, B., and Hagdahl, L., *Experientia*, **3**, 21 (1947).
58. Villee, C. A., Lowens, M., Gordon, M., Leonard, E., and Rich, A., *J. Cellular Comp. Physiol.*, **33**, 98 (1949).
59. Wong, S. Y., *J. Biol. Chem.*, **55**, 427 (1923).
60. Öhmann, L.-O., *Arkiv Zool.*, **36** A, No. 7 (1944).
61. Örström, Å., *Z. physiol. Chem.*, **271**, 1 (1941).
62. —— *Arkiv Kemi, Mineral., Geol.*, **15** A, No. 1 (1942).

ON PROTEIN FRACTIONS AND INORGANIC IONS IN SEA URCHIN EGGS, UNFERTILIZED AND FERTILIZED

SVEN LINDVALL and ASTRID CARSJÖ

The Wenner-Gren Institute, University of Stockholm, Sweden

Received March 5, 1951

The cytoplasm of the sea urchin egg undergoes considerable physical and chemical changes upon fertilization as shown for example by Heilbrunn (3, 4) and by Runnström (13, 14). Mirsky (10) applied more quantitative methods to this study. When extracting frozen and dried eggs with N KCl-solution Mirsky found that a certain portion of the proteins becomes insoluble subsequent to the fertilization of the eggs. Moreover he demonstrated that a corresponding fraction can be precipitated from the extract of unfertilized eggs by ammonium sulfate. These writers (6) have isolated the so called Mirsky protein (1) from the egg of *Strongylocentrotus lividus*. The results agreed with those obtained by Mirsky. Further work showed, however, that the results are rather variable. There seems to be a certain correlation between total N-content of the sample and percentage of soluble N. Even if the preparations are subjected to exactly the same treatment, time and rate of centrifugation etc., the values of total N were found to vary; usually they are about 10 per cent lower in the fertilized as compared with the unfertilized eggs.

MATERIALS, METHODS AND RESULTS

In the present work the eggs of *Echinus esculentus* served as material. The treatment of the eggs was carefully standardized. The eggs were washed repeatedly with sufficient amounts of sea water to swell and gradually remove the jelly coats surrounding the eggs. Thereafter the non fertilized eggs were subjected to a 0.1 per cent solution of commercial trypsin (Merck, Darmstadt). After 5 minutes the eggs were carefully washed with filtered sea water. Upon insemination 70–90 per cent of these eggs were fertilized without formation of a fertilization membrane, see (14). Simultaneously control eggs were inseminated giving 98–100 per cent insemination with a normal fertilization membrane. The fertilized eggs were again washed with sea water or 3 per cent NaCl solution, see for details table I. Thereafter the eggs were centrifuged and frozen in previously cooled Petri dishes at —18° C. The preparations were then dried from the frozen state and the dry powder carefully ground up and stored in an ice box.

The N-content of the frozen and dried material was estimated according to Parnas (12) with the digestion fluid of Arnold and Wedemeyer (see Parnas). In order to evaluate the quantity of sea water or NaCl solution included in the egg samples at the moment of freezing their contents of Na and Cl were estimated. Na was determined in a Beckman flame spectrophotometer subsequent to digestion with H_2SO_4 and perhydrol, Cl according to a micromethod of Rehberg as modified by Schnohr (see Krogh (5)). In this case the digestion was carried out with HNO_3 and H_2O_2 in the presence of Ag NO_3.

The results are summarized in table I. Despite the standardization of the procedure a uniform material was not obtained. The salinity was higher in fertilized eggs with a fertilization membrane than in those in which the vitelline membrane had been removed by trypsin treatment previous to fertilization. The higher salt content in the former case is due to the presence of sea water in the space between the membrane and egg surface (perivitelline space).

Eggs subjected to trypsin treatment were observed to agglutinate subsequent to fertilization. Washing with 3 per cent NaCl-solution brings about a separation of the eggs. The Ca-ions probably favor the stickiness of the cortical material exuded upon the fertilization, whereas this material is liquified when Ca is replaced by Na.

The K, Mg and Ca-contents of the frozen and dried samples were also estimated. Following wet digestion with HNO_3 and perhydrol in quartz tubes the ash was dissolved in a small amount of 2 N HCl. Thereafter Ca was estimated in the flame spectrophotometer and Mg by the method of Drosdoff and Nearpass (2) in which thiazole yellow was used as the reagent. K was estimated in the same way as described above for Na.

The Ca-content of sea urchin eggs has previously been estimated by Mazia (8), Örström and Örström (11) and Monroy-Oddo (9). These authors found much higher Ca-content in their samples than those recorded in table I. The fertilization did not bring about a decrease of the Ca-content comparable to that reported by Örström and Örström and Monroy-Oddo. Conversely the data seem to indicate an increase of the Ca-content upon fertilization of those eggs which were washed with filtered sea water. This apparent increase is due, however, to the sea water taken up by the perivitelline space that is formed upon the elevation of the membrane. Washing with 3 per cent NaCl abolishes this difference completely. The results obtained for K do not differ from those reported by Malm and Wachtmeister (7) for other species.

TABLE I.
Analytical Results

Nr.	Minutes after fertilization frozen	Treatment	Last washing	mg/100 mg dried eggs				γ/100 mg dried eggs		% solubility of total N	
				N	Na	Cl	K	Ca	Mg	in KCl	in H_2O
1U	—	Trypsin	2 times 3 % NaCl	9.50	1.77	2.88	3.35	22.2	110	84.5	85.9
1F1	33	»	» »	9.12	2.44	3.33	3.40	19.4	100	83.0	78.6
1F2	85	»	» »	9.18	2.58	3.63	3.51	22.0	105	86.0	77.4
1F3	150	»	» »	9.02	2.88	3.94	3.36	23.0	95	80.9	75.4
1F4	193	»	» »	9.00	2.91	3.93	3.36	18.0	90	81.3	77.6
2U	—	—	» »	9.55	2.05	3.03	3.71	24.6	100	88.4	76.5
2F1	85	—	» »	8.28	5.47	8.66	3.20	22.7	90	74.0	60.9
2F2	180	—	» »	8.35	5.65	9.10	3.23	24.7	100	71.0	61.0
3U	—	Trypsin	» »	9.30	2.13	3.33	3.65	24.7	105	86.1	81.2
3F1	85	»	» »	9.00	3.13	4.65	3.29	23.9	95	77.5	69.8
3F2	180	»	» »	9.15	2.87	4.00	3.35	20.0	90	82.4	70.6
4U	—	—	Filtered sea water	9.22	1.80	3.17	3.19	31.7	155	88.5	82.0
4F1	95	—	» » »	8.69	4.86	7.32	3 51	82.8	440	70.0	60.6
4F2	130	—	» » »	8.80	3.95	6.29	3.22	54.2	350	71.0	61.2
5U	—	Trypsin	» » »	9.48	1.69	2.83	3.18	27.4	150	88.2	85.9
5F1	95	»	» » »	9.39	2.08	3.15	3.22	28.1	—	87.5	78.1
5F2	130	»	» » »	9.31	2.21	3.24	3.29	30.6	—	85.5	76.3
6U	—	—	1 time in 3 % NaCl	9.61	2.58					87.0	75.8
7U	—	Trypsin	» »	9.46	2.54					86.4	75.4
8U	—	—	3 times in 3 % NaCl	9.25	2.75					84.5	68.4
9U	—	Trypsin	» »	9.44	1.95					85.4	84.0

U = unfertilized F = fertilized

The solubility of the proteins has also been tested in the samples used for the estimations mentioned above. The same proportions between sample and extracting fluid were maintained as in the work of Connors and Sheer (1). The extractions were carried out for one hour at 3° C with distilled water or with N KCl brought to pH 7.2 with phosphate buffer. The solutions centrifuged for an hour at 8 000 × g presented only a feeble opalescence.

The solubility in percentage of the total N-content of the samples is recorded in table I. Fertilized eggs without previous treatment with trypsin give the same decrease in solubility as that found by Mirsky. The pretreated fertilized eggs, on the other hand, show a higher solubility than the non pretreated fertilized eggs. A definite correlation prevails between the solubility and the Na-content of the preparations, as follows from fig. 1.

In measurements of the ultraviolet spectrum of water extracts from both

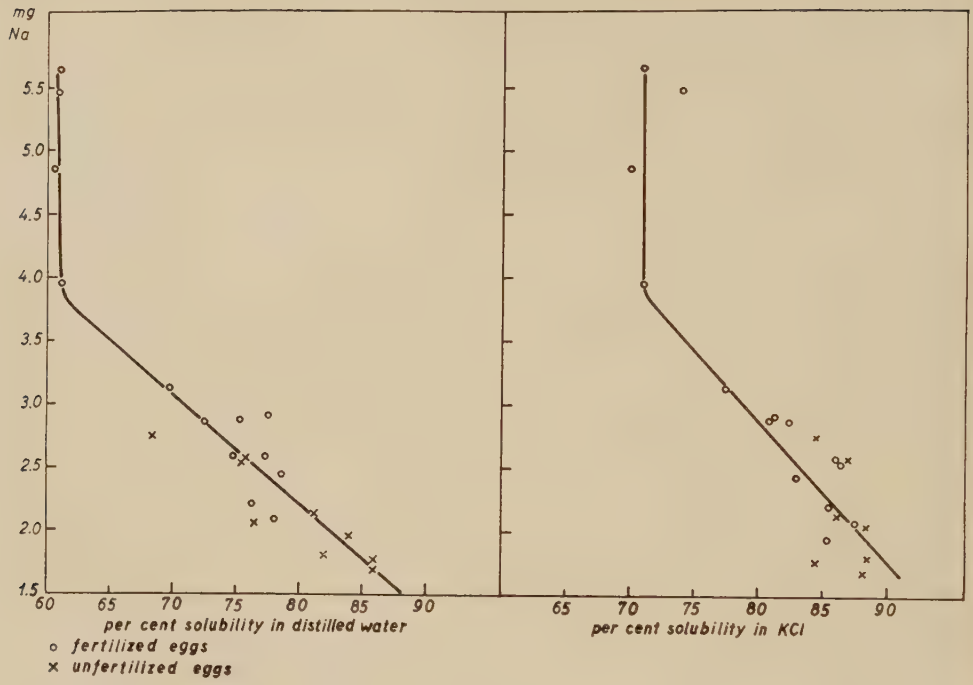


Fig. 1. Protein solubility in relation to Na-content.

unfertilized and fertilized eggs curves were obtained showing an absorption maximum at 270 mμ. Careful dialysis of these extracts against distilled water removed a low molecular component with maximum absorption at 270 mμ. The non dialyzable part on the other hand shows a shift of the absorption maximum to 260 mμ. This holds true, however, only for the unfertilized and for the trypsin pretreated eggs. The extracts of frozen and dried normal eggs with elevated membranes gave no maximum at 260 mμ. The extinction of dialyzed extracts at 260 mμ seems to vary with the solubility of the preparations. When they show a lowered solubility the extinction at 260 mμ decreases until at minimum solubility in distilled water (60 per cent) or in KCl (70 per cent) no extinction at 260 mμ is observed in the extracts.

As previously found one fraction of water extracts of frozen and dried *Strongylocentrotus droebachiensis* eggs is easily precipitated by Ca. This fraction is probably identical with the one which apparently becomes insoluble upon fertilization followed by normal membrane formation. Similar results were obtained with the eggs of *Echinus esculentus*.

Table II
Precipitation with Different Concentrations of Ca.

M $CaCl_2$	0.2	0.1	0.05	0.025	0.0125	0.0062	0.0031	0.0016	0.0008	0.0004
Precipitation	+	++	+++	++++	++++ (+)	+++ (+)	+++	+++	++	+

The optimum of precipitation was determined by adding various concentrations of $CaCl_2$ to water extracts of samples of unfertilized eggs. As follows from table II the maximum precipitation occurs between 0.012 and 0.025 M $CaCl_2$. In similar experiments Mg gives a very slight precipitation with no definite maximum.

A strong precipitation was obtained upon addition of 0.012 M $CaCl_2$ only in the case of unfertilized or trypsin pretreated fertilized eggs. In the fertilized eggs with normal membrane formed on the other hand, no precipitation occurred. After removal of the Ca precipitates the remaining solutions had an N-content corresponding to 58–60 per cent of the total N. This agrees rather closely with the N content found in the water extracts from fertilized eggs with the membrane formed.

A great part of the P present in the extracts was included in the Ca precipitates. The N : P ratio of these was found to be about 9 : 1. The precipitates included the component exhibiting an absorption maximum at 260 mμ.

In certain experiments the distribution of P between the water extract and the non soluble residue was tested. The results showed that the solubility of the P containing substances correlates closely with that of the N. Both extract and residue have been subjected to treatment with Schneider's method (15). It follows from table III that only the nucleic acid P undergoes changes in solubility that parallel the changes in N-solubility. Further data concerning the samples 2U etc. are found in table I. According to Villee *et al.* (17), the desoxyribonucleic acid in the uncleaved egg of *Arbacia punctulata* corresponds only to about 6 per cent of the total nucleic acid—P. The nucleic acid involved in the present experiments is probably the ribonucleic acid.

All the soluble nucleic acid could be precipitated with Ca from water extracts. No precipitation occurs upon addition of Ca to extracts in N KCl-solutions unless the extracts have been dialyzed previously against distilled water.

Mirsky (10) found that freezing and thawing of unfertilized eggs also cause a coagulation of a protein fraction. This probably corresponds to

TABLE III

Analysis of Phosphate Fractions in Sea Urchin Eggs, per cent of Total Phosphorus

Preparation Number		2U	$2F_1$	$2F_2$	3U	$3F_1$	$3F_2$
Water Extract	TCA soluble P	45.5	45.1	45.2	46.1	44.1	45.5
»	Phospholipid P	8.4	8.1	8.1	8.8	9.2	9.0
»	Nucleic Acid P	13.0	2.3	2.7	11.2	3.9	4.9
»	Phosphoprotein P	1.8	1.2	0.9	1.8	1.3	1.4
Residue	TCA soluble P	3.5	3.2	3.0	3.5	3.2	3.5
»	Phospholipid P	17.4	17.6	18.3	16.3	18.5	17.6
»	Nucleic Acid P	8.1	19.4	18.8	10.0	17.0	15.3
»	Phosphoprotein P	2.3	3.1	3.0	2.3	2.8	2.8

the fraction which in his experiments became insoluble upon fertilization. Freezing and thawing of dialyzed water extracts of frozen and dried unfertilized *Echinus* eggs also causes the coagulation of a fraction which has about the same N : P relation as was found in the Ca precipitate. This tends to show that probably the same fraction becomes insoluble in both cases. If the extracts are frozen at —80° C the precipitate was more abundant than when it was carried out at —20° C.

DISCUSSION

Despite the efforts of handling the material uniformly a varying content of NaCl is found even in the trypsin pretreated samples. The salt content is in general higher in the fertilized than in the unfertilized egg samples. This must mean that the former are more elastic. As a consequence they are less densely packed and surrounded by more solution than the latter. This results in a higher salt content in the frozen and dried samples of fertilized eggs.

According to evidence presented the solubility of the proteins depends on the quantity of sea water or salt solution surrounding the eggs at the moment of freezing. A particularly high salt content is found in fertilized eggs with elevated membrane. Here the salts remain enclosed in the perivitelline space. The changes in solubility upon fertilization reported by Mirsky (10) and by these writers (6) is due mainly to this phenomenon, as follows from fig. 1.

The fraction which is rendered insoluble by freezing and drying in the presence of excess of salt seems to correspond to a nucleoprotein. Despite

the varying amounts extracted from different samples the Ca-precipitates of the nucleoproteins have a rather constant N:P ratio, viz. 9:1.

Sevag and Smolens (16) found that streptococcal nucleoprotein cannot be precipitated with Ca when it is liberated from the bacteria by ultrasonic vibrations. The protein and nucleic acid are here linked in a very intimate way whereas upon treatment with acid or alkali only a salt linkage remains. In the latter case the nucleoprotein is precipitable with Ca. The data presented above give no information as to the state of the nucleoprotein in the egg. The Ca precipitability may be due to a secondary change of the nucleoproteins upon freezing and drying. On fertilization or artificial parthenogenesis the egg cytoplasm becomes more strongly basophilic, see e.g. (14, fig. 6). This might be due to a change in linkage between protein and nucleic acid making the latter more accessible to the basic dyes. This would indicate that a real change occurs upon fertilization with respect to the cell component which has been discussed above.

Connors and Sheer (1) found for egg of *Strongylocentrotus* that the "Mirsky protein" contains both pentose and P which points to the presence of nucleic acid. It is therefore surprising that the absorption spectrum of the protein in question did not indicate the presence of this component.

Egg samples which were washed with 3 per cent NaCl-solution do not show any changes in the Ca-content upon fertilization. No uptake into or release of Ca from the egg seem thus to occur. The same result was obtained with respect to Mg.

Örström and Örström (11) and Monroy-Oddo (9) reported that a considerable amount of Ca is given off from the egg subsequent to fertilization. This is probably due to the fact that these authors payed no attention to the jelly coats, which were always carefully removed in the present experiments. Vasseur (17) found that the jelly coat substance binds certain amounts of Ca. Sea water may also be retained in the jelly coats. As Vasseur found the jelly coats tend to fall off more readily from fertilized than from unfertilized eggs, serious errors in the interpretation of the data may arise if these circumstances are not taken into account.

SUMMARY

Frozen and dried unfertilized or fertilized eggs were subjected to extraction by 1-N KCl solution according to the method inaugurated by Mirsky.

In confirmation of Mirsky's results a certain protein fraction seemed to become insoluble in normal fertilization of the egg.

The differences proved almost to vanish if the eggs prior to fertilization were subjected to trypsin treatment which removes the vitelline membrane and thus inhibits the formation of a continuous fertilization membrane.

An obvious correlation was found between the percentage of extracted proteins and the salt content of the egg samples (fig. 1). This is particularly high in frozen and dried fertilized eggs with the fertilization membrane normally formed. This probably accounts entirely for the previously reported differences between unfertilized and fertilized eggs.

Extraction of the frozen and thawed eggs with distilled water gave the same general picture as extraction with N KCl. Here also the solubility of the proteins is correlated with the salt content of the samples.

The dialyzed water extracts gave a strong precipitation upon addition of Ca (optimum at 0.0125–0.025 M). Freezing and thawing of the extracts likewise cause a precipitation of a certain fraction.

The fraction which becomes insoluble in the presence of high salt content of the sample and that precipitated from extracts by additions of Ca or by freezing and thawing seem to be identical. It contains the soluble nucleoprotein of the eggs.

No changes in Ca or Mg content was found to occur upon fertilization of the egg.

This work was carried out under a programme organized by Professor John Runnström. Support was received from the Swedish Natural Science Research Council.

REFERENCES

1. Connors, W. M., and Scheer, B. T., *J. Cellular Comp. Physiol.*, **30**, 271 (1947).
2. Drosdoff, M., and Nearpass, D. C., *Anal. Chem.*, **20**, 673 (1948).
3. Heilbrunn, L. V., Colloid Chemistry of Protoplasm. Protoplasma Monographien 1, Berlin, 1928.
4. —— An Outline of General Physiology, W. B. Saunders Co., Philadelphia and London, 1937.
5. Krogh, A., *Z. vergleich. Physiol.*, **25**, 335 (1938).
6. Lindvall, S., and Carsjö, A., *Arkiv Kemi*, **2**, 293 (1950).
7. Malm, M., and Wachtmeister, L., *Arkiv kemi*, **2**, 443 (1950).
8. Mazia, D., *J. Cellular Comp. Physiol.*, **10**, 291 (1937).
9. Monroy-Oddo, A., *Experientia*, **2**, 1 (1946).
10. Mirsky, A. E., *Science*, **84**, 333 (1936).
11. Örström, Å., and Örström, M., *Protoplasma*, **36**, 475 (1942).
12. Parnas, J. K., *Z. Anal. Chem.*, **114**, 261 (1938).
13. Runnström, J., *Protoplasma*, **5**, 201 (1928).
14. —— *Advances in Enzymol.*, **9**, 241 (1949).
15. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
16. Sevag, M. G., and Smolens, J., *J. Biol. Chem.*, **140**, 833 (1941).
17. Vasseur, E., *Acta Chem. Scand.*, **2**, 900 (1948).
18. Villee, C. A., Lowens, M., Gordon, M., Leonard, E., and Rich, A., *J. Cellular Comp. Physiol.*, **33**, 93 (1949).

THE OCCURRENCE OF MITOSIS IN THE VEGETATIVE PHASE OF BACILLUS MEGATHERIUM[1]

EDWARD D. DeLAMATER and STUART MUDD

Department of Dermatology and Syphilology, and the Department of Microbiology[2], *School of Medicine, University of Pennsylvania, Philadelphia, Pa.*

Received March 6, 1951

INTRODUCTION

During the past fifteen years there has been a rebirth of interest and activity in the study of the cytology and cytochemistry of the bacterial cell. The location, structure and mechanics of the nucleus have attracted particular attention. The extensive review of Meyer (33) admirably presents and summarizes the earlier studies. The more recent reviews of Knaysi (23) and particularly of Lewis (31) synthesize an understandable pattern from the confusing and voluminous literature, while the studies of Robinow (45), Bisset (4), Knaysi (25), Delaporte (14), and particularly Piekarski (42), bring the subject up to date and critically review current concepts concerning the bacterial nucleus and its activities. Because of these recent extensive reviews no effort will be made here to recapitulate the subject in historical detail. However, a brief consideration of recent pertinent work is desirable, in particular in terms of the difficulties encountered and the methods and tools which have been utilized.

Demonstration of the essential structures within the bacterial nucleus has been retarded by certain difficulties which can be stated explicitly in the light of the earlier investigations. The bacterial nucleus is a minute structure whose components are near the limits of microscopic resolution, surrounded by cytoplasm rich in ribose-nucleic acid and its associated protein. The cytoplasmic ribonucleoprotein, by virtue of its chemical and physical properties, tends to mask the desoxyribonucleoprotein components within the nucleus. Techniques hitherto described have not afforded definitive demonstrations of the intranuclear components free from the masking effects of the cytoplasm and in particular of material aggregated about the nuclear desoxyribonucleoprotein. These facts can be shown in clearer relief by brief review of the various methods and tools which have been applied to investigation of the bacterial nucleus. The technique used in the present study permits more definitive demonstration of intranuclear structure.

[1] Paper presented before the VIIth International Congress of Experimental Cytology in New Haven, September 4–8, 1950.

[2] Formerly the Department of Bacteriology.

Light microscopy, because of its general availability and applicability, has constituted the major tool in the study of the cytology of the bacterial cell. The method requires the staining of structures to be visualized. Early staining procedures were not sufficiently differential to afford definition of inherent detail within the bacterial cell. In particular the basophilic staining of the cytoplasm masked the basophilic chromatin structures of the nucleus.

Following the development of the Feulgen technique, Piekarski (39) developed cytologically useful methods which involved acid hydrolysis, as in the Feulgen method, and the use of Giemsa as the stain. Piekarski's method has been used extensively by Robinow (43–45) and, following him, by Klieneberger-Nobel (21, 22), Flewett (15), Bisset (4), Murray, Gillen, and Heagy (38), Delaporte (14), and others, and through its use many of the current concepts of the bacterial nucleus have been arrived at. In general it is felt that the method visualizes essentially the same structures as seen by the Feulgen stain, but produces a denser and, therefore, better seen staining of the nuclear chromatin.

The treatment with warm normal hydrochloric acid utilized in the Feulgen and in the Piekarski-Robinow techniques is conventionally referred to as "hydrolysis" in reference to the hydrolysis of the nuclear DNA which produces the specific staining of the Feulgen procedure. Direct chemical determinations by Vendrely and Lipardy (53), cytological observations by Knaysi (24–26), and electron-microscopic observations by Mudd and Smith (36) have made it clear, however, that a major function of the hydrochloric acid is the extraction of cytoplasmic ribonucleoprotein with its associated basophilia, leaving a residue of DNA and associated materials to give the chromatin staining of the nucleus.

DeLamater, while studying the cytology of yeasts (7, 8), developed a method (6) which also utilizes acid hydrolysis of the cells, but which differs from that of Piekarski by using aldehyde as a mordant, and a single basic dye, basic fuchsin, instead of a mixture of acid and basic dyes (Giemsa). The method produces more permanent and more delicate preparations. This procedure was adapted to the study of the bacterial cell by Smith (47).

Piekarski (39), Robinow (43), and Delaporte (14) used dehydrated balsam mounts of hydrolyzed preparations, and Klieneberger and Smiles (20) and Klieneberger-Nobel (21, 22) used cleared and hardened balsam mounts of unhydrolyzed Bouin-Giemsa preparations. Robinow (44) later stressed the advantages of water mounts over routine dehydration procedures, especially for preparations requiring hydrolysis. Current procedure (Smith (47)) for the most part has utilized water mounts. Such preparations preclude extensive and repeated observation on the same optimal material, and the preparations by their nature fade. Furthermore, fine detail is obscured by the presence of fatty substances which remain in the cell, and by the non-optimal refractive indices of cells mounted in a water medium. It is not intended, however, to deprecate the very important contributions that have been made by these means.

Concerning the application of routine dehydration procedures to bacterial cells, comparison of the results obtained by these methods with those made possible by the freezing-dehydration technique described herein readily demonstrates the marked shrinkage and distortion of the nuclei which occur during routine procedures. To avoid these distortions Delaporte (13) has used water-soluble mounting medium.

Recently DeLamater (9) in conjunction with Blank and McCarthy (5) developed a new freezing-drying technique which for the first time permits the preparation of cytologic mounts of bacteria and other micro-organisms comparable to those utilized for many years by cytologists in other fields. The procedure is simple and inexpensive, and will be presented in detail in the section on methods. In addition to the freezing-dehydration procedure more chemically specific stains are used according to the findings of Atwood and Orinstein (1).

The *phase contrast microscope* has recently been applied to the study of the bacterial nucleus by Tulasne (49) and by Stempen (48). Differentiation of details of pattern in the phase contrast microscope depends largely upon differences in the densities of objects and the effect of these density differences upon the phase of the wave of transmitted light. To date in young, actively growing, gram-negative bacterial cells it has been possible to visualize (48, 49) the nuclear sites or loci as irregular areas of less density than the surrounding cytoplasm, similar to those observed with the electron microscope (17, 36, 37), but is has not yet been possible to define structures within such areas. The chromosomes probably lie within them. The cytoplasmic density apparently depends upon the relative concentration of cytoplasmic ribonucleic acid and associated protein.

DeLamater (10) in unpublished preliminary studies has observed that starvation and/or aging with concomitant reduction of cytoplasmic RNA and, therefore, cytoplasmic density, produced cells in which intranuclear structures became clearly and discretely visible in the phase contrast microscope. Suggestion for such an approach was derived from the work of Knaysi (24, 26). Further studies are in progress.

Ultraviolet illumination has been applied to the study of the bacterial cell in the hope of utilizing the increased resolution obtainable at shorter wave lengths, and also the selective absorption at 2 600 Å by nucleic acids. The first pictures of bacteria published by Köhler (27) in 1904, showed indications of internal structure which, however, cannot be interpreted with certainty. Kruis (28) adapted the Köhler apparatus to micrography of living bacterial cells with monochromatic ultraviolet radiation of 2 750 Å. From the fine pictures obtained he drew the unequivocal conclusion that bacteria contain nuclei which undergo a cycle of division:

> "Die betreffenden Figuren stimmen so vollkommen mit den Kernteilungsfiguren der s.g. einfachen Karyosomkerne überein, indem sie sowohl die durchsichtige Kernsaftzone als auch das in Teilung begriffene, beziehungsweise geteilte Karyosome samt den feinen Verbindungsfäden so deutlich ausweisen, dass über das Wesen und die Bedeutung dieser Strukturen gewiss kein Zweifel bestehen kann."

The resemblance between Kruis' Fig. 4, showing nuclear components of living *Bacillus mycoides*, and the figures of the present communication is striking.

The beautiful pictures of species of the genus Bacillus obtained by Barnard (3), using dark-ground ultraviolet illumination are, indeed, suggestive of nuclei undergoing mitotic division and are so interpreted by him:

> "That bacteria possess a nucleus which undergoes mitotic division is clearly suggested . . ."

The resemblance of structures in Barnard's pictures to the stained mitotic nuclei in the present communication is arresting. Unfortunately further studies of bacteria, utilizing the unique equipment of Barnard, have not been published, so far as we are aware. In the dark-ground views of bacteria smaller than Bacilli, surface diffraction tends to obscure internal detail.

Piekarski (40) in 1938 with the cooperation of Professor Köhler obtained ultraviolet pictures of *Sarcina alba*, showing paired u.v.-absorbing, intracellular structures which were positive to the Feulgen nuclear reaction. These paired structures in Sarcina closely resembled the nuclei later obtained in stained preparations of Staphylococci (19). Intranuclear structures were not resolved, however, in either case.

Wyckoff and Ter Louw (54) used a modification of Barnard's ultraviolet microscope for the study of sporulation in *Bacillus subtilis*. None of the clearly differentiated internal structures were identified by the authors as nuclei.

By far the most detailed investigation of bacteria by means of ultraviolet illumination has been made by Malmgren and Hedén (32), using Köhler's microscope with quartz optical system and the photometric devices of Caspersson. These authors demonstrated a great increase in the cytoplasmic ribonucleic acid during the lag phase, which reached a maximum in the log phase and decreased in the phase of decline. The increase in cytoplasmic nucleic acid was both absolute and relative to the desoxyribonucleic acid. The authors state:

> "In bacteria in the logarithmic phase photometry was possible over any part of the bacteria, as they appeared to be homogeneous from the absorption point of view. On the other hand, in bacteria from the initial culture there were present in the otherwise pale bacterial plasma more strongly absorbing grains, which were avoided during the photometry."

The u.v. absorption in the cytoplasm obviously masked the nuclear desoxyribonucleic acid during the lag and log phases. Magnifications used in this essentially photometric study were insufficient to resolve nuclear components.

The limits of resolution of the *electron microscope* in depicting discrete objects of high density are currently only a few millimicra, an improvement of perhaps 50-fold over the light microscope. Use of this instrument has correspondingly contributed much new detail in the study of the peripheral structures of bacteria, such as cell-wall, plasma membrane (35), flagella (18), and even fine structure on a macromolecular scale (17). Yet in applying the electron microscope to investigation of the bacterial nucleus difficulties similar to those with the light microscope have been encountered. Thus the cytoplasmic nucleoprotein which by its basophilic staining masks the nuclear components, by its electron scattering tends similarly to mask the nuclear components in electron micrographs. Depolymerization of the cytoplasmic ribonucleic acid by ribonuclease (49) largely removes the cytoplasmic basophilia; however, in our experience at least ribonuclease treatment has not appreciably reduced the electron-scattering of the cytoplasm. When the cytoplasmic nucleoprotein is removed by extraction with hydrochloric acid following osmium fixation, the nuclear DNA is aggregated with associated material so that the resulting electron picture is essentially like, and not an improvement over, those previously observed with the light microscope on preparations similarly processed and then stained (36).

Knaysi attacked the problem with great ingenuity by allowing spores of *Bacillus mycoides* to germinate in a medium containing glucose as a source of energy for growth, but deficient in nitrogen and phosphorus. Beautiful pictures of the resulting vegetative cells taken by Baker (26) showed reduced cytoplasmic density and considerable differentiation of internal structures. Nuclear components were still not resolved for reasons not wholly clear. Factors in the situation, however, are the relatively great depth of focus of the electron microscope, which exceeds the diameter of the nucleus, and the requirement that adjacent elements of structure differ sufficiently in density to be differentiated in the electron image. Preparative methods in electron microscopy are at a very early stage of development, and it is to be hoped that means of overcoming current difficulties will be found. Technical factors of preparation and instrumentation in electron microscopy are well discussed by Hillier (16).

In a brief resumé of the methods which have been utilized in the study of the bacterial nucleus, a consideration of the use of *photography* as a tool deserves mention. This is especially so since a certain lack of appreciation of its limitations appears evident (4). In general it may be said that it is possible to photograph what it is possible to see. It must be constantly borne in mind, however, that a photograph is two dimensional. The third dimension microscopically is achieved by means of the focusing mechanism of the microscope. Since the higher the magnification the thinner the optical section, and the more reduced is the third dimension, it should be emphasized that a single photographic record is a record only of a single optical section or plane through an object which is three-dimensional. Observers must guard against the error (4) that interpretation of photographs can be more revealing and productive than extensive study at the microscope. Few will argue that photographic methods, once their limitations are appreciated, are superior to other methods of record for such purposes as the cytologic study of the bacterial cell, if for no other reason than for their relative objectivity in a field where subjective interpretations have been presented in the main in the form of schematic drawings.

Current conceptions of the bacterial nucleus have been so well presented in the reviews already cited that no attempt will be made to recapitulate them here. In summary it may be said that bacteria are now considered to possess clearly demonstrable nucleus-like structures by methods of varying degrees of specificity, and that these nuclei undergo cyclic changes in a divisional process which has not seemed to correspond with classical mitosis. Bacilli are thought to be multinucleate, usually in multiples of two; cocci to be uninucleate.

The process of mitosis, or its equivalent, is as necessary to the biologic stability and activity of bacteria as to the same functions in larger organisms. The present authors feel that the lack of a clear delineation of the structure and activity of bacterial nuclei has not been so much due to the small size of these micro-organisms as to the lack of methods which permit their proper visualization.

The purpose of this paper is to present evidence for the occurrence not only of a true mitotic process in *Bacillus megatherium* but also evidence for the occurrence of a nucleus, the intrinsic structure of which is essentially similar to that long known and accepted as occurring in larger forms. It

might be pointed out in this connection that the nuclei and their mitotic figures recorded here are as large or larger than many long recognized and accepted as occurring in certain fungi (7).

The idea of a true mitotic process in bacteria is startling to bacteriologists only because they have long fostered the concept that the bacteria as a group constitute a unique and distinctive group of micro-organisms. Establishment of the fact that they operate according to the same basic biological (nuclear) mechanisms as other organisms gains importance in the presence of the recent evidence of Lederberg (29, 30) which establishes their operation according to the laws of Mendel.

A preliminary presentation of the occurrence of mitosis in *Bacillus megatherium* has been given by DeLamater (9, 9 a), DeLamater and Mudd (12), and DeLamater and Hunter (11).

Methods.—The organism used in these studies is a strain of *Bacillus megatherium* kindly supplied by Dr. Andrew G. Smith, who had previously obtained it from Dr. C. F. Robinow.

Cytologic preparations for study were made from cultures grown on Morton and Engley medium (34), dextrose nutrient agar, and blood agar (human blood, 5 per cent). Inocula were taken from 18–24-hour-old cultures and incubated on the above media for two hours at 37° C prior to the making of the mounts.

Staining and Dehydration Methods.—The present staining procedure is based upon a finding by Atwood and Orinstein (1) that stains other than pararosaniline in the presence of SO_2 give a specific reaction for an aldehyde radical. Such stains as thionin and azure A give this reaction with aldehyde, but are not decolorized by SO_2. The method is also based on a finding by Blank, McCarthy and DeLamater (5) that it is possible to freeze and simultaneously to dehydrate tissues by chemical means without placing the preparations in a vacuum. These latter procedures have been utilized with modification for purposes of dehydration, and will be described in detail.

Procedure.—From 2-hour-old cultures grown at 37° C, slabs of agar containing the organism are placed upright on a small glass plate and are placed in an atmosphere of osmic acid (0.5 per cent solution) for five minutes. Smaller portions of the agar slab are then cut off and inverted on No. 1 or No. 0 coverslips, and impression preparations are made. These are immediately placed in 1 N HCL at 60° C and hydrolyzed for a period of six minutes. They are then rinsed once in distilled water and placed in the stain, which is made in the following manner: A solution of 0.25 per cent thionin is kept as stock. 10 cc of this is placed in a Columbia staining dish and one drop of thionyl chloride is added (Barger and DeLamater (2)). Preparations are allowed to stain for a minimum of two hours. They are then dipped once in distilled water to remove adhering particles of stain on the preparation. They are then drained on filter paper and transferred to cold absolute alcohol which is kept at —50° C or lower by packing Columbia staining dishes containing the alcohol in solid carbon dioxide (temp. —72° C). The preparations are allowed to remain in this freezing solution for at least

twelve hours. They are then dipped once in absolute alcohol at room temperature, and transferred immediately to xylene. They are then transferred to a second dish of xylene, drained on filter paper, and mounted in one of the mounting resins.

By this chemical freezing-dehydration procedure the bacterial cells are instantaneously frozen in situ. Provided the alcohol is sufficiently cold (has been packed in the CO_2 ice for a sufficiently long time before use), no shrinkage of either the cytoplast or the nucleus is to be observed. Dehydration occurs slowly, and with this there is a penetration of the cell with the cold alcohol, and a gradual fixation and hardening occurs. When the alcohol is not sufficiently cold during this dehydration process, the nuclear elements are markedly shrunken and aggregated into dense balls and masses within clearly defined nuclear vesicles.

Efforts to dehydrate bacterial cells at room temperature by routine techniques have likewise produced marked shrinkage of the nuclear elements, which prohibit the use of such preparations from the cytologic point of view. It is felt that undoubtedly such shrinkage has been misconstrued by past observers, and that the lack of "clearing" and removal of specific Giemsa-staining materials in the water mounts in the preparations of Robinow, Bisset and others prevents clear observations of the minute elements composing the bacterial nucleus.

OBSERVATIONS

The observations and interpretations presented here are based entirely upon the activities of one of us (E. D. DeL.).

The figures demonstrate the sequence of events in the mitotic process of *Bacillus megatherium*. Before describing the results in detail, it should be noted that this organism consists of a multinucleate cell and that each cell contains a minimum of two nuclei, or some multiple of two. The only uninuclear phase which has been observed up to the present time is the spore. The process of sporulation and spore germination will be described in detail elsewhere.

It early became apparent in the study that the axis of the nuclear division occurred at random in relation to the axis of the bacterial cell, and that many of the various nuclear configurations observed were affected by this phenomenon. It also became apparent that shortly after the separation of the chromosomes the axis of the dividing nucleus could bend and thus produce further distortions in the configurations to be observed.

Because of the difficulties in observation which such inherent mechanisms produced, long periods of observation were necessary to obtain the stages which demonstrated the mitotic process. This is especially so since photographic presentation required that the objects to be visualized be in the same optical plane.

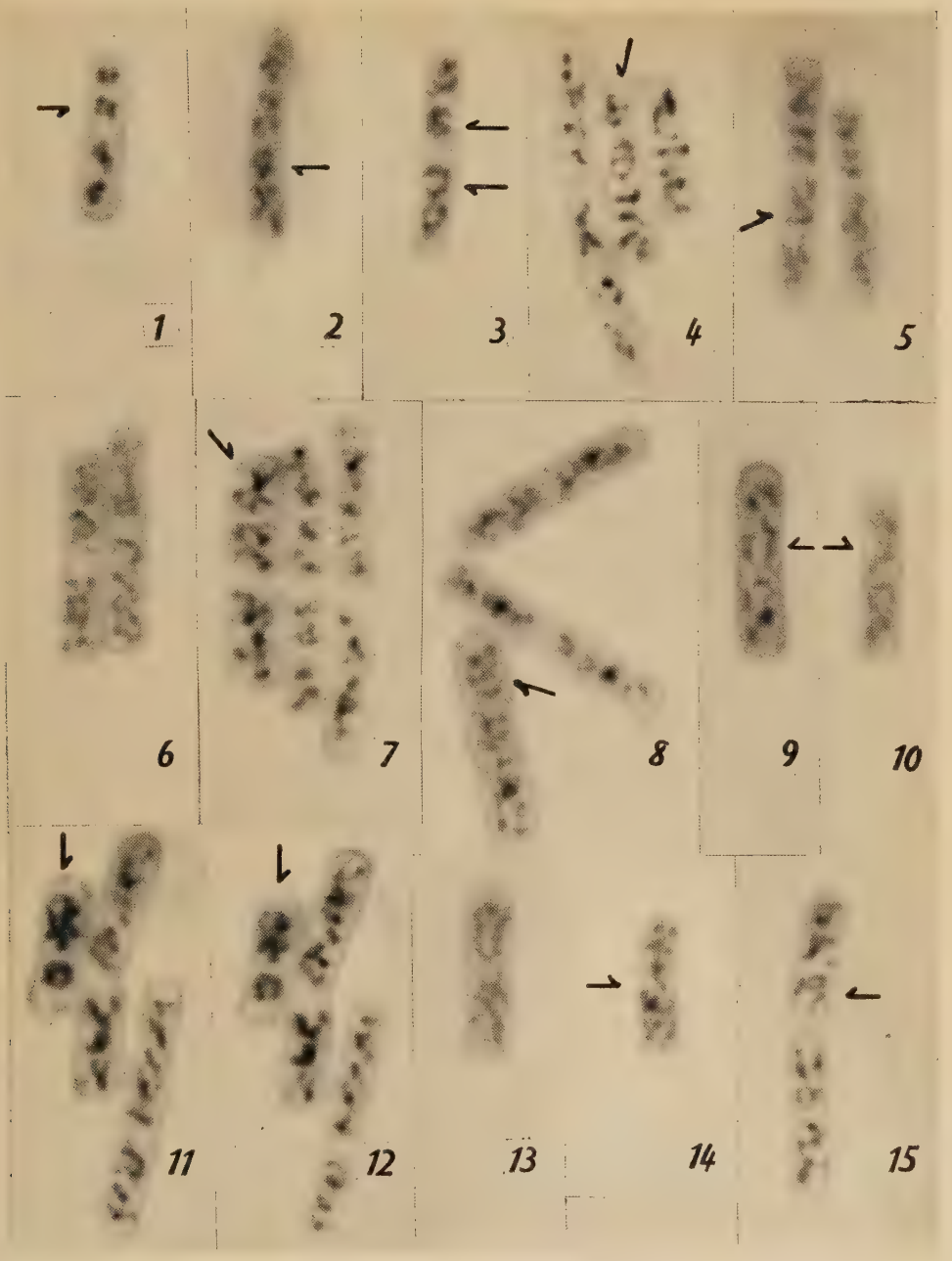
The following sequence of stages have been defined and are demonstrated

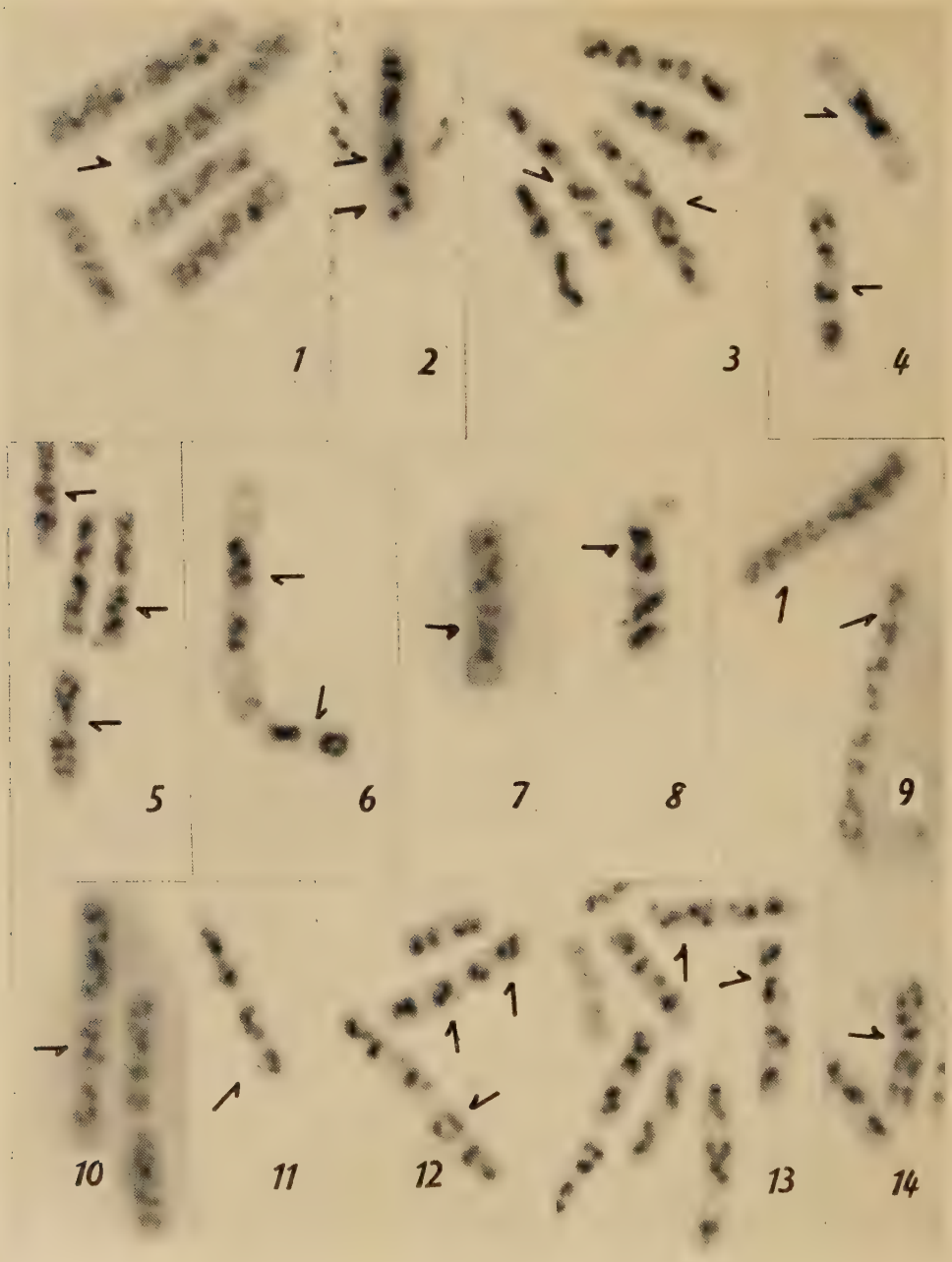
in the accompanying photographs. As the nuclear activity appears to be truly a mitotic one, the various phases of the process will be designated so far as possible with the same terminology defined for the nuclei of larger organisms. Plate I, Figure 1, shows a late telophase of a cell with four nuclei. In the nucleus, indicated by an arrow, three chromosomes are apparent as two rounded granules and a bar. Figures 2–7 show the progressive enlargement of the nucleus and the development of the chromosomes into delicate filaments lying within a discrete nuclear vesicle. Figure 8 shows clearly the limits of the nucleus with the filamentous, beaded chromosomes lying within it. It also clearly delineates the cell membrane and the presence of cytoplasm about the nucleus. Figures 9, 10 and 13 show even more clearly the beaded character of the filamentous chromosomes and what are considered to be rather typical interphase nuclei. Figure 14 is considered to represent an early prophase stage in which the chromosomes are contracting. It demonstrates again the filamentous character of the chromosomes. Figures 11 and 12 are optical sections through the same nucleus, and demonstrate the importance of the orientation of the axis of the nucleus in relation to the axis of the cell. The upper nucleus is longitudinal to the axis

---

PLATE I

Fig. 1. Photo No. 1057 — X4450 — Late telophase. Daughter nuclei have completely separated. Three individual chromosomal units are visible.
Fig. 2. Photo No. 1059 — X4450 — Early interphase. The chromosomes are beginning to elongate into threads.
Fig. 3. Photo No. 1065 — X4450 — Later interphase. Nuclei appear as spheres within which chromosomes are further elongated.
Fig. 4. Photo No. 1075 — X4450 — Later interphase. Nuclear membrane apparent. Chromosomes further elongated into threads.
Fig. 5. Photo No. 1083 — X4450 — Still later interphase stage.
Fig. 6. Photo No. 1007 — X4450 — Large interphase nuclei in which chromosomal threads are clearly apparent.
Fig. 7. Photo No. 1066 — X4450 — Large interphase nucleus.
Fig. 8. Photo No. 1055 — X4450 — Large interphase nucleus clearly surrounded by a layer of cytoplasm between it and the cell membrane. The nuclear membrane is clearly visualized. The chromosomes occur as long threads.
Fig. 9. Photo No. 1076 — X4450 — Interphase stage. Chromosomes in long, beaded threads.
Fig. 10. Photo No. 1037 — X4450 — Interphase chromosomes as long, beaded threads.
Fig. 11. Photo No. 1075 — X4450 — Early prophase. Chromosomes contracting, appear shorter and thicker. This photo is a higher optical plane than the following.
Fig. 12. Photo No. 1074 — X4450 — Early prophase. Lower optical section of same nuclei as shown in Fig. 11. Cross section of chromosomes visualized in lower nucleus of left-hand cell.
Fig. 13. Photo No. 1078 — X4850 — Two very large interphase nuclei. Chromosomes as long beaded threads.
Fig. 14. Photo No. 1065 — X4450 — Early prophase. Three contracting chromosomes clearly defined and lying within nuclear vesicle which is delineated by a bounding membrane.
Fig. 15. Photo No. 1070 — X4450 — Later prophase. Three condensing (shortening) chromosomes in view.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15


1
2
3
4
5
6
7
8
9
10
11
12
13
14

of the cell while the lower nucleus is perpendicular to it. In the upper nucleus the three chromosomes are clearly shown in Figure 11, and less clearly in Figure 12. In Figure 12 in the lower nucleus, cross-sections of the chromosomal filaments can be seen. The stages presented in these figures are also thought to be early prophase, as are those in Figure 15 in which the chromosomes are found shortened or contracted in anticipation of division.

In Plate II, Figure 1, in the indicated cell can be seen what is interpreted as the early centrosome. In the nucleus second from the left and in the terminal nucleus, two granules can be seen which are interpreted as the divided centrosome. It is thought that these two granules represent the polar bodies of the incipient nuclear division. The nuclei demonstrated here are considered to be in early prophase. In Figure 2, Plate II, is shown a cell with four nuclei, all of which are in the metaphase stage of division. The lowest nucleus in the cell seen from polar view shows the three metaphase chromosomes as two rounded masses and a bar. The adjacent nucleus demonstrates a typical metaphase spindle with the two polar bodies, or

---

PLATE II

Fig. 1. Photo No. 1050 — X4450 — Prophase. The two left-hand nuclei in the indicated cell show associated granules. There is one granule next to the right-hand nucleus (above and to the right), and two next to the left-hand nucleus, lying to the left in the tip of the cell. These are interpreted as centrioles.

Fig. 2. Photo No. 1033 — X4450 — Metaphase spindle showing the chromosomes on the metaphase plate between two distinct polar bodies (upper nucleus). The lower nucleus, viewed through the poles, shows the three chromosomal granules as two round granules and a bar.

Fig. 3. Photo No. 1019 — X4450 — Metaphase spindle showing chromosomes on plate between polar bodies. Right-hand cell indicated shows early interphase nuclei.

Fig. 4. Photo No. 998 — X4450 — Lower cell shows telophase nucleus with chromosomal cluster migrating toward polar body. Upper cell shows condensed anaphase of what appears to be a diploid cell. (This process will be described and documented separately.)

Fig. 5. Photo No. 1083 — X4450 — Telophase stages showing separation of chromosomes and their migration toward the polar bodies. The three chromosomes are apparent in several instances.

Fig. 6. Photo No. 1007 — X4450 — Anaphase-telophase divisional stages.

Fig. 7. Photo No. 1061 — X4450 — Anaphase-telophase separation of chromosomes.

Fig. 8. Photo No. 1016 — X4450 — Anaphase and telophase divisional stages. Chromosomal masses dense.

Figl 9. Photo No. 1059 — X4450 — Telophase. Connecting chromosomal thread shows in right-hand nucleus.

Fig. 10. Photo No. 1059 — X4450 — Telophase stages. Chromosomes migrating to poles. Polar bodies still evident.

Fig. 11. Photo No. 996 — X4450 — Late telophase. Daughter nuclei separated. Cluster of chromosomes opposite polar body. Position of polar body and chromosomes shows rotation and bending of divisional axis commonly observed.

Fig. 12. Photo No. 1001 — X4450 — Late telophase, early interphase nuclei. Chromosomes separating. Nuclear vesicle reforming.

Fig. 13. Photo No. 1008 — X4450 — Late telophase–early interphase stages.

Fig. 14. Photo No. 1003 — X4450 — Early interphase nuclei showing chromosomes as discrete round granules and rods.

centrosomes, at the two poles, and with the chromosomes lying on the equatorial plate. This figure also demonstrates the importance of the relationship of the axis of the dividing nucleus to the axis of the cell. Figure 3 shows another example of a metaphase figure with the two polar bodies present. In the right-hand cell of this figure the nuclei have begun to enlarge following the telophase stage of the division. In Figure 4 is shown a peculiar anaphase stage of division, further interpretation of which will be presented elsewhere. It was at first thought that this figure represented a stage in the vegetative cycle. Work done subsequent to these studies indicates, however, that this photograph may represent an additional phase in the growth of the organism. In the lower cell in Figure 4 is shown a late telophase of a typical vegetative nucleus in which can still be seen the polar body in the second nucleus from the bottom of the cell. Figures 5, 6 and 7 show anaphase-telophase stages in the division. In the lower cell in Figure 5 the axis of the division is again important in formulating an interpretation. In the lower portion of the lower nucleus the three chromosomes can again be defined. In Figure 8 the upper nucleus as indicated is dense, but the delicate fibril connecting the dividing nuclei can be seen. This stage is interpreted as telophase. In Figure 9, which appears to be a somewhat later stage, the chromosomal granules can be made out in the telophase stages shown, and in the right-hand cell the delicate fibrils connecting the dividing chromosomes can still be observed. In Figure 10 is clearly shown a telophase stage in which the polar bodies of both daughter nuclei are still evident. This is also apparent in the lowest nucleus shown in Figure 11. Figures 12, 13 and 14 demonstrate later stages in the reorganization of the daughter nuclei into vesicles within which the chromosomes lie. It is thought that at this stage the nuclear membrane is re-established about the chromosomes. Persistent polar bodies can still be made out in the right-hand cell of Figure 13, and the chromosomes can be seen to be separating and becoming more clearly defined as the nuclei enlarge in Figure 14.

COMMENT

The various configurations observed in what appear to be normal divisional stages can be confusing because of the variation of their axes and positions and the degrees of aggregation of their intrinsic chromosomal units. Moreover, it must be constantly kept in mind that one is working at the limits of resolution of the instruments available, and that the hazards of misinterpretation in consequence are large. Recognition of and compensation for

the difficulties and hazards of this type of work can, however, be made to increase the accuracy of the observations made.

Current studies (using the same methods as herein) on several additional organisms, including other members of the genus *Bacillus, Escherichia coli,* a *Micrococcus,* and *Caryophanon,* indicate that a true mitotic process operates in these organisms also. These studies will be presented in detail in the future.

The present study indicates that current concepts concerning the nature of the bacterial nucleus and its included chromosomes must be modified and extended to include the presence of a distinct nuclear membrane and the elongation and contraction of the chromosomes during the vegetative and divisional cycle. Bisset's "condensed" and "vesicular" nuclei may fit into the cycle as herein described. Further, it would appear that chromosome numbers of various bacterial species now become subject to analysis.

It is felt that the intrinsic structure of the bacterial nucleus in all its phases, as indicated by this study, shows its essential similarity to the nucleus of larger organisms. These findings would tend to bring the bacteria cytologically into the general biologic economy where they already are biochemically, and where they certainly belong, and give pause to those who would consider them (i.e., bacteria) as a unique group of organisms.

Establishment of a true mitotic process in bacteria further aids in the formulation of the basic mechanisms of variation, mutation, genetic recombination and segregation, all of which are known to operate in this group of organisms. Studies currently in progress are aimed at these general problems from a combined cytologic, genetic and biochemical point of view.

CONCLUSION

Evidence is presented for the occurrence of a true mitotic process in *Bacillus megatherium* comparable to that which is known to occur in larger organisms. Intrinsic nuclear structure also appears to be similar.

REFERENCES

1. Atwood, K., and Orinstein, L., *Exhibit A.A.A.S., New York City* (abstract in program, 1949).
2. Barger, J. D., and DeLamater, E. D., *Science,* **108**, 121 (1948).
3. Barnard, J. E., *A System of Bacteriology in Relation to Medicine, Med. Res. Council, London Privy Council,* **1**, 115 (1930).
4. Bisset, K. A., The Cytology and Life History of Bacteria, The Williams and Wilkins Co., Baltimore, Md., pp. 1–136, 1950.

centrosomes, at the two poles, and with the chromosomes lying on the equatorial plate. This figure also demonstrates the importance of the relationship of the axis of the dividing nucleus to the axis of the cell. Figure 3 shows another example of a metaphase figure with the two polar bodies present. In the right-hand cell of this figure the nuclei have begun to enlarge following the telophase stage of the division. In Figure 4 is shown a peculiar anaphase stage of division, further interpretation of which will be presented elsewhere. It was at first thought that this figure represented a stage in the vegetative cycle. Work done subsequent to these studies indicates, however, that this photograph may represent an additional phase in the growth of the organism. In the lower cell in Figure 4 is shown a late telophase of a typical vegetative nucleus in which can still be seen the polar body in the second nucleus from the bottom of the cell. Figures 5, 6 and 7 show anaphase-telophase stages in the division. In the lower cell in Figure 5 the axis of the division is again important in formulating an interpretation. In the lower portion of the lower nucleus the three chromosomes can again be defined. In Figure 8 the upper nucleus as indicated is dense, but the delicate fibril connecting the dividing nuclei can be seen. This stage is interpreted as telophase. In Figure 9, which appears to be a somewhat later stage, the chromosomal granules can be made out in the telophase stages shown, and in the right-hand cell the delicate fibrils connecting the dividing chromosomes can still be observed. In Figure 10 is clearly shown a telophase stage in which the polar bodies of both daughter nuclei are still evident. This is also apparent in the lowest nucleus shown in Figure 11. Figures 12, 13 and 14 demonstrate later stages in the reorganization of the daughter nuclei into vesicles within which the chromosomes lie. It is thought that at this stage the nuclear membrane is re-established about the chromosomes. Persistent polar bodies can still be made out in the right-hand cell of Figure 13, and the chromosomes can be seen to be separating and becoming more clearly defined as the nuclei enlarge in Figure 14.

COMMENT

The various configurations observed in what appear to be normal divisional stages can be confusing because of the variation of their axes and positions and the degrees of aggregation of their intrinsic chromosomal units. Moreover, it must be constantly kept in mind that one is working at the limits of resolution of the instruments available, and that the hazards of misinterpretation in consequence are large. Recognition of and compensation for

the difficulties and hazards of this type of work can, however, be made to increase the accuracy of the observations made.

Current studies (using the same methods as herein) on several additional organisms, including other members of the genus *Bacillus*, *Escherichia coli*, a *Micrococcus*, and *Caryophanon*, indicate that a true mitotic process operates in these organisms also. These studies will be presented in detail in the future.

The present study indicates that current concepts concerning the nature of the bacterial nucleus and its included chromosomes must be modified and extended to include the presence of a distinct nuclear membrane and the elongation and contraction of the chromosomes during the vegetative and divisional cycle. Bisset's "condensed" and "vesicular" nuclei may fit into the cycle as herein described. Further, it would appear that chromosome numbers of various bacterial species now become subject to analysis.

It is felt that the intrinsic structure of the bacterial nucleus in all its phases, as indicated by this study, shows its essential similarity to the nucleus of larger organisms. These findings would tend to bring the bacteria cytologically into the general biologic economy where they already are biochemically, and where they certainly belong, and give pause to those who would consider them (i.e., bacteria) as a unique group of organisms.

Establishment of a true mitotic process in bacteria further aids in the formulation of the basic mechanisms of variation, mutation, genetic recombination and segregation, all of which are known to operate in this group of organisms. Studies currently in progress are aimed at these general problems from a combined cytologic, genetic and biochemical point of view.

CONCLUSION

Evidence is presented for the occurrence of a true mitotic process in *Bacillus megatherium* comparable to that which is known to occur in larger organisms. Intrinsic nuclear structure also appears to be similar.

REFERENCES

1. Atwood, K., and Orinstein, L., *Exhibit A.A.A.S., New York City* (abstract in program, 1949).
2. Barger, J. D., and DeLamater, E. D., *Science*, **108**, 121 (1948).
3. Barnard, J. E., *A System of Bacteriology in Relation to Medicine, Med. Res. Council, London Privy Council*, **1**, 115 (1930).
4. Bisset, K. A., The Cytology and Life History of Bacteria, The Williams and Wilkins Co., Baltimore, Md., pp. 1–136, 1950.

5. Blank, H., McCarthy, P. L., and DeLamater, E. D., *Stain Technol.* (in press) (1951).
6. DeLamater, E. D., *Stain Technol.*, **23**, 161 (1948).
7. —— *Trans. New York Acad. Sci., Ser. II*, **11**, 162 (1949).
8. —— *J. Bact.*, **60**, 321 (1950).
9. —— *Stain Technol.* (in press) (1951).
9a. —— Abstract. meetings Natl. Acad., Sci, Wash., O, C, *Science* **113**, 477 (1951).
10. —— Unpublished observations.
11. DeLamater, E. D., and Hunter, M. E., *Amer. J. Bot.* (in press) (1951).
12. DeLamater, E. D., and Mudd, S., *Fifth International Congress Microbiol.*, Rio de Janeiro, Aug. (1950). *International Society for Cell Biology*, VIIth International Congress, New Haven, Sept. (1950).
13. Delaporte, B., Year Book, Carnegie Institution, Washington, D. C., p. 166, 1948–49.
14. —— *Advances in Genetics*, **3**, 1 (1950).
15. Flewett, T. H., *J. Gen. Microbiol.*, **2**, 325 (1948).
16. Hillier, J., *Annual Review of Microbiol.*, **4**, 1 (1950).
17. Hillier, J., Mudd, S., and Smith, A. G., *J. Bact.*, **57**, 319 (1949).
18. Houwink, A. L., and van Iterson, W., *Biochim. et Biophys. Acta*, **5**, 10 (1950).
19. Hunter, M. E., Mudd, S., and Woodburn, M. A., *J. Bact.*, **60**, 315 (1950).
20. Klieneberger, E., and Smiles, J., *J. Hyg.*, **42**, 110 (1942).
21. Klieneberger-Nobel, E., *J. Hyg.*, **45**, 410 (1947).
22. —— *Q. J. Microscop. Sci.*, **91**, 340 (1950).
23. Knaysi, G., *Botan. Rev.*, **4**, 83 (1938).
24. —— *J. Bact.*, **55**, 753 (1948).
25. —— Elements of Bacterial Cytology, Comstock Publ. Co., Inc., 2nd Ed., Ithaca, N. Y., pp. 1–375, 1951.
26. Knaysi, G., and Baker, R. F., *J. Bact.*, **53**, 539 (1947).
27. Köhler, A., *Z. wiss. Mikroskop.*, **21**, 273 (1904).
28. Kruis, K., *Ceska Akad. Prague, Bull. Internat., Classe Sci., Math., Natur., Méd.*, **18**, 141 (1913).
29. Lederberg, J., *Heredity*, **2**, 145 (1948).
30. —— *Annual Review of Microbiol.*, **3**, 1 (1949).
31. Lewis, I. M., *Bact. Revs.*, **5**, 181 (1941).
32. Malmgren, B., and Hedén, C.-G., *Acta Path. Microbiol. Scand.*, **24**, 417 (1947).
33. Meyer, A., Die Zelle der Bakterien, Gustav Fischer, Jena, 1912, pp. 1–285.
34. Morton, H. E., and Engley, F. B., Jr., *J. Bact.*, **49**, 245 (1945).
35. Mudd, S., and Anderson, T. F., *J. Am. Med. Assoc.*, **126**, 561 (1944).
36. Mudd, S., and Smith, A. G., *J. Bact.*, **59**, 561 (1950).
37. Mudd, S., Smith, A. G., Hillier, J., and Beutner, E. H., *J. Bact.*, **60**, 635 (1950).
38. Murray, R. G. E., Gillen, D. H., and Heagy, F. C., *J. Bact.*, **59**, 603 (1950).
39. Piekarski, G., *Arch. Mikrobiol.*, **8**, 428 (1937).
40. —— *Zentr. Bakt. Parasitenk. I, Orig.*, **142**, 69 (1938).
41. —— *Zentr. Bakt. Parasitenk. I, Orig.*, **144**, 140 (1939).
42. —— *Ergeb. Hyg. Bakt. Immunitätsforsch. Exptl. Therap.*, **26**, 333 (1949).
43. Robinow, C. F., *Proc. Roy. Soc. B.*, **130**, 299 (1942).
44. —— *J. Hyg.*, **43**, 413 (1944).
45. —— Addendum: Nuclear apparatus and cell structure of rod-shaped bacteria, In The Bacterial Cell, by R. J. Dubos, Harvard Univ. Press, Cambridge, Mass., pp. 355–377, 1945.
46. Robinow, C. F., and Cosslett, V. E., *J. Applied Phys.*, **19**, 124 (1948).
47. Smith, A., *J. Bact.*, **59**, 575 (1950).
48. Stempen, H., *J. Bact.*, **60**, 81 (1950).
49. Tulasne, R., *Compt. rend. Soc. Biol.*, **143**, 1390, 1392 (1949).
50. Tulasne, R., and Vendrely, R., *Compt. rend. Soc. Biol.*, **141**, 674 (1947).
51. —— *Nature*, **160**, 225 (1947); **161**, 316 (1948).
52. Vejdovsky, F., *Zentr. Bakt. Parasitenk. II Abt. Allgemeine*, **6**, 577 (1900); **11**, 481 (1904).
53. Vendrely, R., and Lipardy, J., *Compt. rend. Acad. Sci.*, **223**, 342 (1946).
54. Wyckoff, R. W. G., and Ter Louw, A. L., *J. Exptl. Med.*, **54**, 449 (1931).

A METHOD FOR MEASURING SMALL RETARDATIONS OF STRUCTURES IN LIVING CELLS[1]

SHINYA INOUÉ[2]

Department of Biology, Princeton University, Princeton, New Jersey

Received March 17, 1951

The measurement of birefringence in living cells is accompanied by special difficulties not encountered in birefringence measurements of ordinary crystals. The retardation is very small (usually 0.1–5 mμ), and there are a great number of light scattering particles in the cell which make the birefringent image difficult or impossible to discern. Besides, the cross section of the birefringent object in the cell is usually elliptical or circular, and the area with uniform strength of birefringence is very small. Furthermore, the birefringence at the edge of the object changes gradually because of the shape of its cross section, and makes the measurement even more difficult. For these reasons, the ordinary compensation method, which can be used for measuring the retardation of thin crystals very sensitively (see Jerrard 3, for a summary of compensators), is not suitable for accurate measurement of the retardation of organelles in the cell.

Swann and Mitchison (5) have used densimetry on photographs of spindles in dividing cells to avoid the above mentioned difficulties. Their method is laborious and exacting, but will undoubtedly prove useful to some, and sufficiently accurate if precaution is taken to subtract the intensity differences caused by factors other than birefringence.

In the following is described a new direct method (which may be called the 'double half-shade method') for measuring retardation in complicated objects with small retardations.

In figure 1, 'A' is an object whose cross section is an ellipse. This is placed between crossed nicols with its axes 45° to the axes of the nicols. Another small crystal 'B' is placed beside the object with its axes also in the 45°

[1] This research was supported in part by funds of the Eugene Higgins Trust allocated to Princeton University.

[2] Charlotte Elizabeth Procter Fellow. Present address: Department of Anatomy, School of Medicine, University of Washington, Seatle 5, Washington.

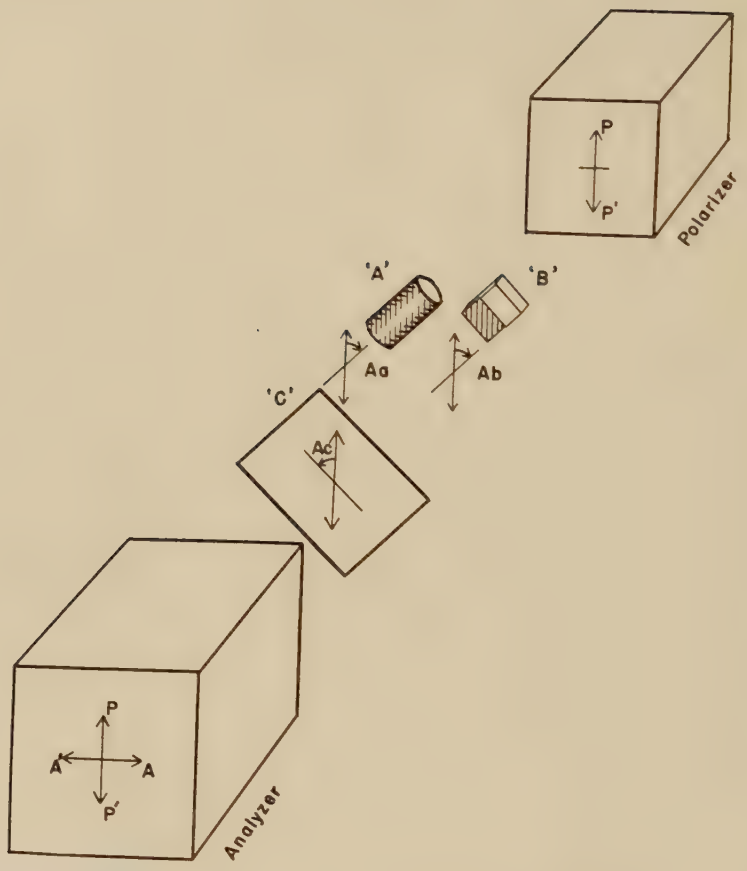


Fig. 1. Arrangement of the optical elements in the double half-shade method.

position. Part of this crystal is slightly thicker, and therefore its retardation slightly larger than the rest. In addition to the two, another large crystal 'C', the compensator, is placed as shown in the figure, so that each of the light beams which passes through the object and the small comparator crystal also passes through the compensator.

First, let us assume that the retardation of a particular part of the object and the comparator crystal are the same, and their axes parallel. If we rotate the compensator (C) under this condition, the particular part of the object and the comparator will go through extinction simultaneously. Furthermore, if the compensator is rotated a little further, a small white area will

begin to appear simultaneously at the center of the object and on the thicker side of the comparator. In this way, even if light scattering objects are superimposed upon the birefringent object, *by matching the change of the intensity* of the two, it is nevertheless possible to match the birefringence of an object with the birefringence of a comparator. Thus, knowing the retardation of the comparator, one can determine the retardation of the object.

Instead of using a comparator crystal which has exactly the same retardation as the object, a crystal with a somewhat higher retardation could be used by rotating it around an axis parallel to the optical axis of the measuring device. The apparent retardation of the crystal can, in this way, be made equal to the retardation of the object as shown in the following calculation.

Let the retardation of the object, the comparator and the compensator be *Ra*, *Rb* and *Rc* respectively, each of their axes making an angle *Aa*, *Ab* and *Ac* with the polarizer axis, (fig. 1). Consider both the retardation of the object and the apparent retardation of the comparator to be equal to that of the compensator, but with an opposite sign. This brings both the object and the comparator into extinction with the compensator. The retardations R are all sufficiently small so that their sines can be equated to the retardations expressed in radians. Then from Fresnel's equations, as Köhler (4) has shown (also see Bear and Schmitt, 1),

$$\sin(2\,Aa) \times \sin(Ra/2) = -\sin(2\,Ac) \times \sin(Rc/2)$$

and also

$$\sin(2\,Ab) \times \sin(Rb/2) = -\sin(2\,Ac) \times \sin(Rc/2).$$

Therefore,

$$\sin(2\,Aa) \times \sin(Ra/2) = \sin(2\,Ab) \times \sin(Rb/2).$$

Since the object is lying in the 45° position, $\sin(2\,Aa)$ is 1, and so

$$Ra/2 = \sin(2\,Ab) \times Rb/2$$

This equates the retardation of the object with the apparent retardation of the comparator. Therefore, if the retardation (*Rb*) of the comparator is known, the retardation (*Ra*) of the object can be determined by measuring the angle *Ab*. At this angle, the comparator goes through extinction and becomes brighter again simultaneously with the object when the compensator is rotated around the extinction position.

The approximation used in this calculation will lead to an error of not

greater than 1 percent, if the retardations of the object, comparator and compensator are each less than a thirtieth of a wave length.

In applying the described method to measurements of retardation of small objects under the microscope, it is almost impossible to prepare a comparator crystal which can be placed side by side with the object, and further to place it so that it can be rotated around its axis. The actual method is, therefore, to project an image of the comparator crystal into the object plane. The image of the comparator crystal can then be focused and compared side by side with the object.

The crystal is a thin piece of mica about 0.2–0.3 mm square, mounted at the center of a rotating drum whose angle of rotation can be measured with a vernier to a twentieth of a degree. This is placed in front of the polarizer located immediately in front of the field lens (that is, the front lens of the light source), with its image focused by the condenser, onto the plane of the object. For the present arrangement, the polarizer would have to be placed approximately 20 cm away from the condenser. Also, neither a mirror nor a prism should be used in between the polarizer and the condenser since it would disturb the condition of polarization, and so the whole microscope including the light source should be arranged on an optical bench. A microscope arranged in this fashion will be described in another paper.

The compensator can be placed at any suitable position between the polarizer and the analyzer since there are only two retardation plates in any one pathway of light. If there were three or more retardation plates, in general their positions are not inter-changeable (3, 2), and so for an accurate measurement, the lenses, slide and cover glasses etc. involved in the system should be perfectly strain free.

It has been possible to measure the retardation of spindle components to a tenth of a mμ by the method described. This has proven to be very useful in tracing the change of retardation of the spindle which is disintegrating under the influence of colchicine, or for measuring the form birefringence curve of the fixed spindle.

The method is general and accordingly of wide applicability. It has notable advantages over the photographic method, since it automatically excludes the effect of intensity differences of the image caused by factors other than birefringence, (such as absorption, refraction, scattering etc.) and because the measurements can be done very rapidly, with great accuracy and high precision.

SUMMARY

A new half shade method for measuring retardations in optically complicated structures is presented. By using a polarization microscope with an extinction of 10^5, a retardation as small as a tenth of a mμ (in objects not larger than a micron in diameter) can be measured very rapidly by this method. Especially important is the fact that such measurements can easily be made without injury, directly upon the living cells.

REFERENCES

1. Bear, R. S., and Schmitt, F. O., *J. Optical Soc. Am.*, **26**, 363 (1936).
2. Hsü, H. Y., Richartz, M., and Liang, Y. K., *ibid.*, **37**, 99 (1947).
3. Jerrard, H. G., *ibid.*, **38**, 35 (1948).
4. Köhler, A., *Z. wiss. Mikroskop.*, **38**, 29 (1921).
5. Swann, M. M., and Mitchison, J. M., *J. Exptl. Biol.*, **27**, 226 (1950).

COMPARATIVE STUDIES OF MOUSE TUMORS WITH RESPECT TO THEIR CAPACITY FOR GROWTH AS "ASCITES TUMORS" AND THEIR AVERAGE NUCLEIC ACID CONTENT PER CELL

GEORGE KLEIN

The Wallenberg Laboratory for Experimental Cytology at the Institute for Cell Research, Karolinska Institutet, Stockholm, Sweden and the Institute for Cancer Research, Philadelphia, Pa[1].

Received May 31. 1951

In a previous paper (22) some aspects have been summarized of the use of the Ehrlich ascites tumor for certain studies on the growth, chemical composition, and morphology of tumor cells and for studies concerning the effect of physical and chemical influences on these three qualities. From previous literature and from our experiments the conclusion was drawn that the Ehrlich ascites tumor presents several advantages over solid tumors for certain types of work, mainly because of its being a comparatively homogeneous and rather concentrated suspension of free tumor cells among which, according to Brodersen (2) and in conformity with our experience, degenerative changes are relatively uncommon. This suspension form makes possible inoculation of counted numbers of cells, repeated withdrawal-punctures during the whole tumor development, and, after a certain stage, chemical determinations of cellular constituents where the amount of substance found can be related to the cell number.

It has been shown (12) that at 257 mμ in the ultraviolet microscope the ascites tumor cells show a comparatively uniform and high absorption which is in contrast to the highly variable absorptions in solid tumors, as found by Caspersson and Santesson (3). This comparative uniformity of absorption, probably depending on uniformity of nutrition in all cells, makes this material especially suitable for cytochemical determinations of e.g. nucleic acids, where the number of cells to be measured depends on the variability of the material. Direct contact of injected chemicals with the cells makes chemotherapeutic experiments independent of the vascular system of transplanted tumors (27). On the other hand, simultaneous testing of chemicals on the solid and ascites forms can help in clarifying

[1] Present address: Karolinska Institutet, Stockholm 60, Sweden.

the mechanism of action, whether it is directed only against the vascular system, or against the tumor cells proper.

On the basis of these and other advantages it was felt necessary to carry out a systematic study on representative tumor material to decide the question of whether the phenomenon of growth as a "nearly pure culture" of dissociated tumor cells is limited to the Ehrlich carcinoma or whether it has a more general significance. Should it be possible to obtain positive results, a variety of tumors could be used in the same way as the Ehrlich ascites tumor and help to approach tumor cell growth and chemistry problems quantitatively. It was considered also necessary to obtain such tumors, as far as possible, in inbred strains of mice.

In a previous work (24) E. Klein and the author have shown that the transformation of a solid transplantable mouse carcinoma was possible in this way, but only after a number of successive intraperitoneal transfers of the exudate which developed during the growth of this solid tumor in the peritoneal cavity. This fluid at first contained very few tumor cells and only after a number of transfers has the percentage of tumor cells increased to high values. The relationships between solid growth on the peritoneum and free tumor cell occurrence in the fluid have been studied for this tumor and also for the Ehrlich ascites tumor.

Occurrence of free tumor cells in neoplastic exudates is a well known phenomenon which accompanies growth of tumors on the serous surface itself. This has been described already more than 80 years ago (31) and has since been the subject of many investigations (19, 40, 50, 52). The term ascites tumor, as it was applied in the German literature to the Ehrlich ascites tumor (2, 27, 45) and recently by Yoshida (51) to a rat sarcoma described by him, denotes a special case namely a condition where there is an exudate accumulation in the peritoneal cavity containing a high number of free tumor cells, which sometimes approaches a "nearly pure culture" (51). Systematic studies on the production of such a condition with many different tumors are not available. The most important contributions are those of Loewenthal and Jahn on the Ehrlich ascites tumor (30), that of Yoshida on his rat sarcoma (51) and that of Goldie and Felix on Sarcoma 37 and a lymphoma in dba mice (13). In the case of Yoshida the condition apparently arose by chance and accompanied the solid growth of the respective tumor in the peritoneal cavity. Yoshida could not reproduce the condition with other tumors. Loewenthal and Jahn, Goldie and Felix have produced it experimentally. Goldie and Felix whose results have been communicated to the author after the present work

was already in progress, studied in detail some growth characteristics of the free peritoneal cells. They found a rather different behaviour in the case of the two tumors they tested. This will be discussed later.

In the present series of experiments a wider range of tumor material has been tested as to its behaviour after intraperitoneal transplantation.

Some preliminary results have already been given in a previous note (23). Here, the following questions will be approached:

1) The possibility of transforming solid tumors into typical ascites tumors. It is realized that the term ascites tumor is not an adequate one. It will be maintained because of its extensive use in the German literature but needs exact definition as to its meaning. As previously (24), it will be applied to neoplasms growing in the form of free and homogeneously distributed viable tumor-cells in the ascitic fluid, in cases, where the frequency of tumor cells as compared to other cell elements is after a certain stage of development, very high, and approaches a pure culture. A certain time after inoculation there is always some solid infiltration present, too, and by modification of different variables, such as inoculated cell number, cell viability, animal strain, it is possible to shift the ratio between solid growth and free cells in the fluid so that solid growth predominates and the peritoneal fluid contains mainly inflammatory cells. Such a condition will not be called an ascites tumor. The term ascites tumor will thus not mean a certain particular tumor or some particular tumors under all conditions of growth in the peritoneal cavity, but a certain particular *form* of growth with the restrictions named above, in which form certain tumors under certain conditions are able to grow. The tumors were so chosen as to represent different morphological types in different strains of mice.

2) The possibility of differential counting of tumor and non-tumorous cells in the peritoneal fluid in the case of the tumor material used.

3) Comparisons between the free tumor cells obtained in the peritoneal fluid in the case of the different tumors as to their morphological appearance, viability, mitotic index, etc.

4) Correlations between inoculum size, survival time and extensiveness of solid growth.

5) Comparisons between morphology of the respective solid tumors which were used for the experiments and those produced by reinoculation of the developed ascitic fluids after a number of ascitic fluid transfer generations.

6) Average pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) content per cell in the fluids, where a sufficiently high concentration of tumor cells was attained.

EXPERIMENTAL[1]

I. *Materials*

Young adult mice of both sexes, with a weight of 20–25 grams were used. Tumors which were not strain specific, were kept in white mice obtained from a commercial farm. For the tumors which originated in inbred strains, mice of the corresponding inbred strain or their F1 hybrids were used. These strains were the Strong A, Strong C3H, C 57 black, C 57 leaden, dba and AKM. Most of the inbred mice have been obtained from Dr. T. S. Hauschka to whom our sincere thanks are due. A number of the animals have been obtained from the Roscoe B. Jackson Memorial Laboratory. The Yoshida sarcoma was kept in 3 months old non-inbred rats of both sexes. All animals were kept on a constant diet. The following *tumors* were used:

a) Carcinoma

1) TA3. This tumor, which was obtained from Dr. T. S. Hauschka at the ICR in Philadelphia, has originated as a spontaneous mammary adenocarcinoma in a female A mouse. It was kept then by subcutaneous transfers in A mice, and showed a gradual loss of differentiation during subsequent transfers. The material used for the present experiments was taken from an 8-day old tumor of the 16th transfer generation. On histological examination the material showed to be a poorly differentiated adenocarcinoma. The tumor was found by Hauschka to take and kill in 100 per cent of over 400 strain A mice or AF1 hybrids.

2) Tumor 15091a was obtained from the Roscoe B. Jackson Laboratory at Bar Harbor through the courtesy of Dr. E. Fekete. This tumor originated in 1928 in a female of the A strain and was diagnosed as a medullary adenocarcinoma of the mammary gland (4). It was found to grow in 100 per cent of strain A mice. After a number of transfers it became more and more anaplastic, and since 1935 it was stated to be a spindle cell carcinoma, subcutaneous transplants of which kill the hosts in 21–24 days. The material used was an 11-day old subcutaneous transplant, which upon histological examination showed to be a completely anaplastic tumor with polymorphous cells.

3) Tumor ICR, which was obtained from Dr. I. Diller, originated as a spontaneous mammary carcinoma in a female of the C3H strain at the Institute for Cancer Research in 1948. When obtained, it had passed through over 50 subcutaneous transplant generations, took in about 80 per cent of C3H mice and killed them in about 1 month. The material used was a 28-day old transplant, which was histologically a medullary carcinoma.

4) Tumor EO 771 was obtained from Dr. K. Sugiura at the Sloan-Kettering Institute. It has originated at the Roscoe B. Jackson laboratory in Bar Harbor in 1940 as an adenocarcinoma of the mammary gland in a C57 black mouse, foster-

[1] The following abbreviations are used: FTG: fluid transplant generation, number of intraperitoneal transfers of ascitic fluids. MST: median survival time. PNA: pentosenucleic acid. DNA: desoxypentosenucleic acid.

nursed by a C3H foster mother. It was stated to grow in 100 per cent of C57 black line 6 mice and to kill them in about 3 weeks. The material used was an 11-day old transplant, which upon histological examination showed the picture of a cell-rich medullary carcinoma.

5) Data and references about carcinoma Krebs 2 are to be found in a previous work (24). In the present work the ascites tumor obtained from the earlier investigations was used which has passed over 20 ascites generations. Originally this tumor was a carcinoma simplex of either the skin or the mammary gland (49) in a hybrid male mouse.

6) References and data concerning the Ehrlich ascites tumor are given in a previous paper (22). In the present work our own strain was used which had been derived from a solid Ehrlich carcinoma (obtained from Dr. A. Fischer in Copenhagen) in 1948 and since then passed over 150 transplant generations as an ascites.

7) Tumor C 954, which was obtained from Dr. Fekete in Bar Harbor, originated in 1940 as a spontaneous carcinoma of liver parenchyma cells in a C57 leaden mouse. It was stated to take in 100 per cent in the strain of origin and kill the hosts in 5–8 weeks. 14-day old subcutaneous transplants were used for the present experiment. Histological examination showed a fairly anaplastic hepatic carcinoma.

8) Transplantable hepatoma 98/15 in C3H mice was obtained from Dr. G. H. Hogeboom at the National Cancer Institute. This tumor arose spontaneously in a male C3H mouse and was described by Edwards et al. (11). It was shown to be a highly differentiated, slowly growing hepatoma. For the present experiment, a 32-day old transplant was used, which showed the typical histological picture.

The following spontaneous carcinomas were tried:

9) A spontaneous tumor of the mammary gland arising in a female of the A mouse strain obtained from Dr. I. Diller. It will be called SPAD. On histological examination it appeared to be an adenocarcinoma with partly acinar and partly solid medullary structure and with a number of hemorrhagic cysts.

10) Another spontaneous mammary tumor similarly in a female A mouse was obtained from Dr. Hauschka and will be called SPAH. Histologically, it was very similar to the previous tumor with somewhat better differentiated glandular formations and smaller and fewer cysts.

11) A spontaneous mammary tumor in a female C3H mouse (SPC3H) was obtained from Dr. Diller. This was also a highly differentiated adenocarcinoma with glandular and cystic formations, hemorrhages and intracystic papillary growth.

12) A spontaneous mammary tumor in a female dba mouse was obtained from Dr. Hauschka (SPdba). It was a well differentiated adenocarcinoma.

b) Sarcomas.

13) Tumor MC1A, obtained from Dr. E. E. Miller, was a rhabdomyosarcoma induced in 1945 by methylcholanthrene in a C3H mouse (34), and kept by subcutaneous transfers since then. It was stated to take in 100 per cent of C3H mice, and it was carried over 50 serial transplant generations. Its rate of growth was relatively slow, reaching a size of 1×1.5 cm in about 24 days. The material used

for the present experiment was a 30-day old transplant, which upon histological examination, proved to be a rhabdomyosarcoma with numerous giant cells.

14) Tumor MC1E, obtained from Dr. Diller, was induced as No. 13 and was a fibrosarcoma, 100 per cent pathogenic in C3H mice and killing in 4–5 weeks. 30-day old transplants were used, which histologically showed the picture of a fibrosarcoma.

15) Tumor MC1M, obtained from Dr. Miller, belongs to the same group of induced tumors as 13 and 14. It started out originally as a rhabdomyosarcoma in 1946. It was pathogenic for C3H mice, killing them in about 4–5 weeks. Our material was a 28-day old transplant, which histologically was an anaplastic polymorphous-cell sarcoma.

16) Tumor MC1N was a similarly induced fibrosarcoma, obtained from Dr. Diller. Material from a 23-day old transplant was used which showed the picture of a polymorphous cell sarcoma.

17) Sarcoma T241 was obtained from Dr. Sugiura. This tumor arose in a C 57 black mouse following local application of dibenzanthracene. It was a rapidly growing tumor, stated to attain the diameter of 10 mm in one week. Histologically, it was a fibrosarcoma.

18) Sarcoma 37 was obtained from Dr. Diller. This tumor originated at the Imperial Cancer Research Fund in London. It was kept by subcutaneous transfers in hybrid mice. The material used for the present work was an 8-day old transplant, showing the histological picture of an anaplastic, polymorphous-cell sarcoma.

19) Crocker sarcoma 180 has been obtained from Dr. Diller. It has originated in 1914 at the Crocker Institute. It was kept in Swiss mice, and killed them in 4–5 weeks. A 10-day old transplant was used for the present work, which was histologically an anaplastic sarcoma.

20) The Wagner osteogenic sarcoma was obtained from Dr. Sugiura. This tumor occurred spontaneously in the Sloan-Kettering colony of AK mice (46). No osteopoesis was observed in the particular tumor obtained, which was a 16-day old transplant.

21) The Yoshida ascites sarcoma of the rat has been secured in its ascites form from Prof. Tomizo Yoshida. All details concerning this neoplasm are to be found in Yoshida's paper (51). It has been kept as an ascites tumor since it originated in 1943.

c) Lymphomas.

22) Lymphosarcoma 6C3HED was received from Dr. Fekete in Bar Harbor. This tumor originated at Dr. Gardner's laboratory in a female C3H mouse treated with equiline benzoate. It was stated to take in 100 per cent of C3H mice which survived 3–4 weeks (29). The material used was a 10-day old subcutaneous transplant, which infiltrated diffusely the surrounding subcutaneous tissue and was histologically a lymphoma.

23) A transplantable lymphoma, originally arisen as a thymus tumor in a dba 212 mouse at the National Cancer Institute, has been obtained from Dr. Dalton. This tumor was identical with the one which Goldie and Felix used for their studies of the growth characteristics of free tumor cells in the peritoneal fluid (13). It was requested and obtained in November 1950 after the work with all other tumors

was already in progress and when the author then visiting the National Cancer Institute was informed about the work of Dr. Goldie. The tumor has been kept in dba x C hybrids. The material used belonged to a line which always has been kept as a solid tumor and was not passed through the peritoneal cavity previously. 10-day old subcutaneous transplants were used. Histology: Lymphoma.

24) The Patterson lymphosarcoma originated spontaneously in an AKM mouse at the Sloan Kettering Institute and was obtained from Dr. Sugiura. A 16-day old subcutaneous tumor was used, which infiltrated flatly the surrounding subcutaneous space. Histology: lymphoma.

25) The Krebs-Rask-Nielsen-Wagner lymphosarcoma was obtained from Prof. C. Krebs (Aarhus). This tumor originated in a hybrid white mouse in 1928 at Krebs' laboratory. It was previously found to be able to grow as an ascites tumor (26). The materials used were 10–14-day old subcutaneous transplants, infiltrating diffusely the surrounding connective tissue and showing the histologic picture of a malignant lymphoma.

d) Other tumors.

26) Tumor C 1300, which was obtained from Dr. Fekete, was found by Dr. Cloudman at the Jackson Memorial Laboratory in 1940. It was originally "a huge irregular red and white tumor filling the abdominal cavity of an A strain mouse." This tumor was described by Gorer as "a round cell tumor, possibly a neuroblastoma" (14). 11-day old subcutaneous transplants were used. Histologically they showed the picture of a diffusely growing anaplastic tumor, with mainly polyhedral and round cells.

27) The Harding Passey melanoma was obtained from Dr. Sugiura. The origin of this tumor has been described in detail (16). It occurred as a spontaneous growth on the ear of a stock mouse in 1925. It was found to be a slowly growing tumor on transplantation, taking 2–6 months to reach a size when it could be retransplanted. It was found to take in 90 per cent of stock mice. The material used by us was a 60-day old transplant, in an AKM mouse. It was an entirely black tumor, histologically showing the typical picture described by Harding and Passey.

28) Melanoma S 91 was described by Cloudman (5). It originated spontaneously in 1937 in a female dba mouse at the base of the tail. The material obtained from Mr. Hankwitz at the Institute for Cancer Research was a 70-day old transplant, histologically showing the picture of a melanotic melanoma.

II. *Methods*

The solid tumors used for inoculation were thoroughly minced, suspended in 0.9 per cent NaCl and injected intraperitoneally in amounts of 0.5–1.0 ml. If the tumor originated in an inbred mouse strain, the strain of origin or corresponding F1 hybrids were always used for inoculation.

Two groups of animals were inoculated with the solid tumor masses each containing 6 animals. One group was used for observing survival time. The second group was kept until the animals showed marked abdominal distension and 4–6 animals from the combined groups died. The rest of the animals in the second

group was killed then. It has been found that in cases, where survival times were generally lower than 30 days, this point of time falls within the close neighbourhood of the median survival time. After the skin had been removed, a small opening was cut on the abdominal wall and all available fluid was collected with a syringe or a Pasteur-pipette. Smear preparations of the fluid or, in cases of very dilute fluids, of the sediment after centrifugation were immediately prepared and treated according to Papanicolaou's method (48). The peritoneal cavity was then examined and solid tumors and gross infiltrations were noted. Histological specimens were prepared routinely from a great number of different areas of the parietal and subdiaphragmal peritoneum, the mesentery of the duodenum including the pancreas, and parts of the intestines and organs covered by the visceral peritoneum.

Where a fluid accumulation was found in the peritoneal cavity, this fluid was used for further intraperitoneal inoculation. During this procedure the policy was followed that large amounts of pooled fluids from different animals were to be inoculated, thus, in general 1.0–2.0 ml per animal. If the amount of available fluid permitted, 10 animals were inoculated. If there was no fluid available, the experiment has been repeated in some cases, and, if fluid still did not appear in appreciable amounts, the tumor was discarded.

Half of the animals inoculated with the fluid were observed as to survival time, the other half were used for further inoculation, in the same way as presented above for the first transplant generation. If fluids again were available, they were used for further transplantation in 0.4 ml amounts, and this method of always transferring 0.4 ml amounts of the fluids was then followed during all subsequent generations. At each sacrifice of animals, smears and histological material was prepared in the same way as named above. From the third *fluid transplant generation* (FTG in the followings) the fluid was also inoculated subcutaneously into 2–3 animals and the solid tumor developed was compared histologically with the original solid tumor.

In certain cases, and mainly with fluids containing high concentrations of tumor cells, viability tests were performed. Dead and living cells were counted partly on microcinematographic films after preparing the material as described previously (21, 22), and partly by the method of Schrek (44). The two methods showed a fairly good agreement and therefore, for routine purposes, the simple Schrek method was used. Results will be given on page 541.

III. *Differential counting of cell types in the peritoneal fluid*

a) *Normal peritoneal fluid.*

0.1–0.2 ml amounts of peritoneal fluid were withdrawn with a capillary glass pipette from 5 groups of mice, which belonged to the C3H, Strong A, C57 black, C57 leaden and dba strains, respectively. Each group consisted of 5 male mice. Wet smears were immediately fixed and stained according to the method of Papanicolaou. The occurring cell types have been carefully studied and differentially counted. In all smears, 500 cells have been counted. In general, the same classification was applied as by the Vincent Memorial Laboratory Staff (48) for normal cells of peri-

toneal fluids. There was no certain strain specificity to be found in the cellular composition of peritoneal fluids, the individual variations in all strains being great. The fluids were, however, most often belonging to one of two main types: one with high concentrations of lymphocytes, and the other with predominantly mesothelials and histiocytes. Both types occurred in all 5 strains. On the whole, the following ranges resp. means were found for the different cell types: mesothelial cells 7–58 per cent (32.0); histiocytes 0.6–61 per cent (23.1); polymorphonuclears 0–2 per cent (0.34); lymphocytes 15–92 per cent (50.0); and mast cells 0–3 per cent (0.55). The variations for mesothelial cells, histiocytes and lymphocytes are considerably greater than those found by Goldie and Felix in the case of 12 CFW mice (13). Figs. 11–15 show some microphotographs of peritoneal fluids in different animals.

b) *Sterile peritonitis.*

Sterile peritonitis was produced in 3 groups of 7–10 mice in three different ways. First, 0.5–1.0 ml Omnadin (Bayer) was injected similarly to the experiment of Seeger (45). Secondly, suspensions of "Kieselguhr" (finely ground diatomaceous earth) were injected. This was chosen because, as found by Jones and Rous (18), it produces damage to the peritoneal surface which is followed by proliferation of the mesothelium and submesothelial connective tissue. Finally, cellfree ascitic fluid from the Ehrlich ascites tumor has been injected to a third group. This group was at the same time the control group in an experiment which is reported on page 542, and the changes in the composition of the peritoneal fluid after the injection are shown in Fig. 8. All three groups were punctured at regular intervals after the injections and the fluids examined as in the case of the normal peritoneal fluids. All three substances elicited a qualitatively similar though quantitatively very different reaction. After an initial appearance of high numbers of polymorphonuclear leukocytes, their number decreased and mainly mesothelial cells and histiocytes predominated, which again decreased later on. The reaction in the case of Omnadin and ascites serum was over in 2–3 days, while in the case of Kieselguhr it lasted 6–10 days. In the case of the Kieselguhr injections, the reaction was also much more pronounced, and after 3–4 days, there was a number of mesothelial cells present, which showed signs of cellular activity, thus, increased nucleoli, polymorphism, mitotic figures, etc. The mitotic figures, however, never appeared in a frequency higher than 0.5 per cent of all mesothelial cells present. Abnormal mitoses were not observed. Figs. 16–17 show fields of these fluids.

Thorough study of the morphology of the cell types in all fluids treated in paragraphs a) and b) served as a basis for the differential counting of cells in the neoplastic exudates.

c) *Neoplastic exudates.*

The fluids were withdrawn and treated in the same way as those under a) and b).

1) Ascites tumors Ehrlich and Krebs 2.

The examination of smears from well-developed ascites fluids, withdrawn around median survival time, revealed the presence of great numbers of free cells, which were decidedly different from any of the cells observed in normal or inflammatory peritoneal fluids examined. These cells were predominating and very similar to

each other in the case of both tumors examined. They varied in size quite considerably, but in general, they were larger or as large as the largest cells observed in non-tumorous exudates. Their most distinguishing feature was the nucleus, which was large, contained several heavily stained chromatin clumps and one or several large nucleoli. The cytoplasm was often heavily basophilic. (Figs. 27, 28, 30.) In the ultraviolet microscope at 257 mμ the cells showed a massive absorption (Fig. 50). No cell complexes could be observed. The differences from normal mesothelial cells were only quantitative and thus concerned cell size, nuclear size, amount of chromatin, nucleolar size and cytoplasmic basophilia. These differences were however so marked that very few borderline or doubtful cases could be seen in these fluids. Mitotic figures were common, and abnormal mitoses were often to be seen. For reasons to be mentioned in the first part of the discussion, these cells were supposed to be identical with free tumor cells and will henceforth be referred to as T cells.

The relative frequency of T cells as a percentage of all cells present depends on a number of factors.[1] It is correlated with at least the following 4 factors studied: number of viable cells inoculated, time after inoculation, mouse strain, and damaging influences on the cells. The first factor has been investigated in a previous work (24), where it has been found that low cell numbers which produce long and variable survival times with much solid tumor formation, will cause a higher frequency of non-tumorous exudate cells in the developing ascitic fluids than injection of high cell numbers. This is true for the Ehrlich and Krebs 2 tumors as well. The same thing happens if the cells inoculated are damaged either before injection by storage e.g., or during their growth by chemicals, as acriflavine e.g. (22). In all cases, the increased frequency of non tumorous cells was found to be connected with increased and more variable survival times and an increase in the amount of solid infiltrations and tumors in the peritoneal cavity. Fig. 7 shows that time after inoculation has an effect upon the composition of the peritoneal fluids in the case of the Ehrlich ascites tumor. Similar findings were made on the Krebs 2 tumor. Finally, different mouse strains seem to react differently to the growth of these tumors. Variations were found in the frequency of non tumorous exudate cells around median survival time in groups of mice belonging to different strains injected with the same number of cells from the same pool. The A strain, C57 black strain and C57 leaden strain behaved rather similarly and have shown non-tumorous cell frequencies in the range of 8.2–23 per cent. In the dba

[1] It is of course realized that the differentiation of T cells in terms of exact counts is open to criticism because of the lack of *specific* criteria for tumor cells. Nevertheless, a quantitative attempt — even with the admitted possibility of certain errors — seems to be a definite advantage over merely recording impressions.

strain, the range was 19.2–29.0 per cent and in the Swiss strain 13.4 per cent was the lower limit, but most of the animals that developed ascites at all, showed a very high frequency of non tumorous cells, often reaching an almost complete lack of detectable tumor cells. Solid tumor development without exudate formation and spontaneous regressions occurred too. All these values refer to the Ehrlich tumor and injection of about 20×10^6 cells. The differential countings have been performed on 7–20 animals.

The frequency of non tumorous cells in the Ehrlich ascites tumor has also been studied by Koprowska and Koprowsky (25) at Papanicolaou's laboratory. They found 31 per cent non tumorous cells in 11 Swiss mice (range 7–52 per cent) and 26 per cent in A mice (9–42 per cent). They used however a different subline of the tumor and the number of inoculated cells and time of examination of the fluids were not identical with those used by the author.

Some factors determining the frequency of inflammatory cells detected in the ascitic fluid have also been studied in the case of the Yoshida ascites tumor by Saito et al. (41). They found a dependence on rat strain and on the "proliferation grade of tumor cells," to which the host reaction is "inversely proportional." These findings are essentially in agreement with our experience with the Ehrlich and Krebs 2 ascites tumors.

Dependence of non-tumor cell frequency upon the time after inoculation have also been found by Yoshida in the case of the Yoshida tumor (51) and by Goldie and Felix in the case of their lymphoma in dba mice (13).

It seems to be advisable for comparative experiments to inoculate cell numbers which are sufficiently high as to cause small variations in survival time; to collect the ascitic fluids around median survival time and to use always the same strain of mice. In our previous works, where ascites tumors were used for nucleic acid determinations (12, 22) these criteria have been followed with the exception of keeping the mouse strain constant. The average frequency of non tumorous cells in the Ehrlich ascites tumor was then found to be 10.4 per cent with a range of 2 to 17 per cent. Hybrid mice were used. A recent reinvestigation on our present hybrid mouse material has shown somewhat higher frequencies with a mean of 18.2 per cent (range 7–24 per cent). To some degree, this difference is due to increasing experience in differentiating non tumorous cells, but this does not entirely account for it. Probably, differences in the genetic constitution of the hybrids played a role, too.

2) Exudates produced during the growth of different carcinomas and sarcomas in the peritoneal cavity.

Differential diagnosis of T cells has been attempted on the basis of the experience with the Ehrlich and Krebs 2 ascites tumors. Only cells showing markedly dif-

ferent characteristics from normal exudate cells were counted as T cells. In general, the changes from normal exudate toward T cell have been taking the same directions as reported above for the ascites tumors (see e.g. Figs. 23–25, 32–34, 36–38). However, the morphological appearance of the T cells has been different with different tumors. These differences will be mentioned in connection with the corresponding tumor. In general, differential counting of T cells was relatively easy at times when they occurred in high frequency, and became increasingly difficult in the case of low frequencies. The smears of exudates containing low frequencies of T cells have been re-examined after an exudate of the same tumor with high frequency was obtained, where the T cells of the respective tumor could be studied in detail.

3) Exudates produced during the growth of lymphomas in the peritoneal cavity.

This group has to be treated specially because of the small sized lymphoblast-like neoplastic cells (Figs. 43, 45, 47). The differential diagnosis of these cells from some normal constituents of the peritoneal fluid, such as small mesothelial cells, plasma cells, big lymphocytes, etc., has been found to be much more difficult than in the case of the huge and hyperchromatic cells from the other different types of tumors studied. There was a number of dubious cells occurring which could be present in frequencies of 5–15 per cent. Doubtful cells have been omitted from the differential countings, but even so, the counts are less reliable and involve greater error than in the case of the other tumor types studied, the cell types of which have a character entirely foreign to normal or inflammatory peritoneal fluid. That such counts have still been possible, was mainly due to the fact that the fluids in question were very uniform in general and contained high numbers of typical lymphoblast-like cells.

IV. *Growth of the tumors tested in the peritoneal cavity*

a) Carcinomas.

1) TA3. Fig. 1 shows the changes in approximative median survival time and tumor cell frequency around median survival time in the ascitic fluid during 13 transplant generations with the ascitic fluids (FTG). Ascitic fluid was available in most animals of all generations in amounts of 0.5–5 cc. The fluids in the early generations were bloody, and no tumor cells or tumor cell complexes could be detected except in a few dubious cases. During FTG 6-7 a marked change was taking place. At the same time as median survival times decreased, there appeared a number of obvious tumor cells in the fluids, partly freely, and to a greater part in solid cell complexes. In the following generations their number increased strikingly, both of the complexes and of the free cells. In fig. 1 the percentage of tumor cells reprecents only the free T cells and cell complexes so small that the number of T cells they contained could be counted. Thus, a great number

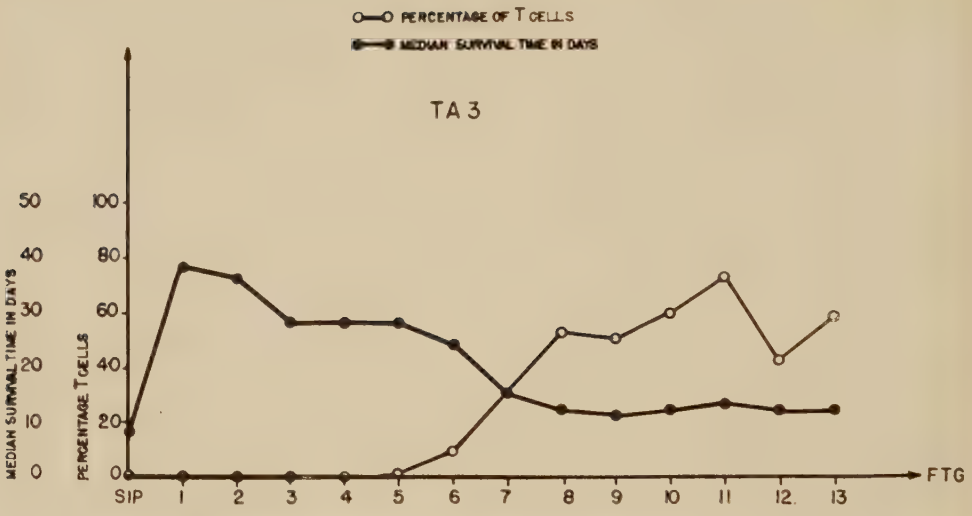


Fig. 1. Relationship between approximative median survival time in days and average percentage of free T cells (and T cells in small cell complexes) found in the ascitic fluid at median survival time ± 4 days (1–6FTG) or ± 2 days (SIP and 7–13 FTG) during 13 fluid transplant generations (FTG) of carcinoma TA3. SIP: intraperitoneal inoculation of the solid tumor.

of T cells present in the large complexes is neglected. Still, the general tendency is well illustrated. In all generations there was solid infiltration of the peritoneal cavity present. Huge solid tumors were only seen in the early generations. The free T cells were very characteristic for this tumor and neither similar to normal mesothelial cells nor to the cell types of the Ehrlich ascites tumor. The cells were smaller than the Ehrlich cells, they were round and contained one or several huge nucleoli. (Figs. 18–21.) The ascitic fluids were hemorrhagic on all generations, but more regularly and more intensely so in the earlier than in the later ones.

2) 15091a. Fig. 2 shows the approximative median survival times and T cell frequencies during 14 FTGs. If compared to TA3, the curves show the same general trend, but this is less marked. Another important difference is that the frequency of detectable T cells is not zero in the earliest FTGs, but it is around 10 per cent. Furthermore, the appearance of the T cells was also very different from TA3. There was no marked tendency for complex formation except in a very few cases, but the cells were building a uniform suspension. The T cells showed great variations in size, generally they were larger than the largest mesothelial cells, and bi- and multinucleate cells were frequent. All T cells were distinguished by an

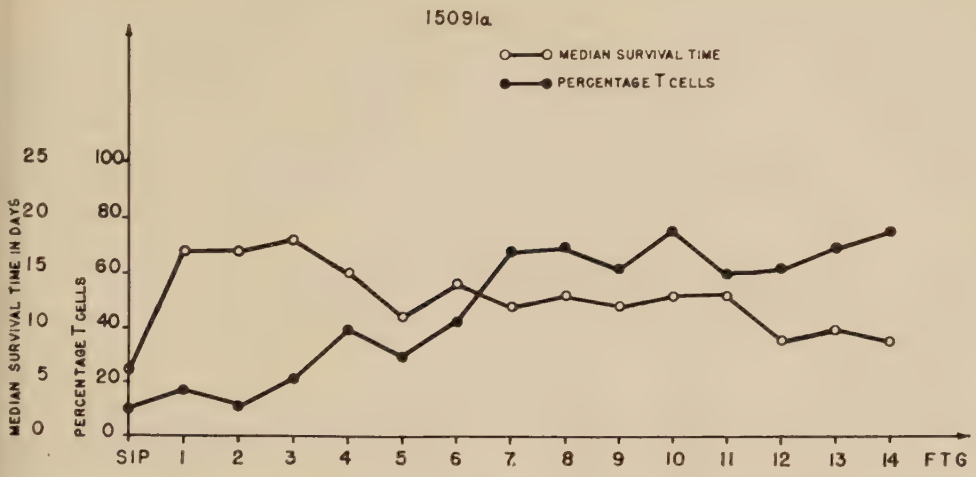


Fig. 2. Relationship between approximative median survival time in days and average percentage of free T cells found in the ascitic fluid at median survival time ± 2 days in 14 fluid transplant generations (FTG) of carcinoma 15091a. SIP: intraperitoneal inoculation of the solid tumor.

abundant chromatin, which was present in large clumps, big nucleoli, and strongly basophilic cytoplasm. (Figs. 22–25.) The fluids were always grossly hemorrhagic. Gross infiltration of the entire peritoneal cavity was always present, but its amount tended to decrease in later generations. Compact solid tumors appeared only up to and including the fourth FTG. The ascitic fluids often showed a tendency toward ready clotting. Frequency of mitotic figures around median survival time was 1.13 per cent (average of 7 samples) of all T cells present.

3) All animals inoculated with tumor ICR developed solid tumors, accompanied by a hemorrhagic exudate. Their median survival time was 28 days. No T cells could be detected in the exudates. The exudates were injected into ten mice in the usual way, using pooled exudates from different animals. However, all animals thus injected survived observation period of three months without tumor or ascites development.

4) Tumor EO 771 gave, after i.p. inoculation, solid tumors in 8 out of 10 animals. 2 animals developed hemorrhagic exudates with no detectable T cells. The tumor was carried through only 4 FTGs, during which solid growth and exudate formation occurred, with no or, in the last two generations, 1–6 per cent free tumor cells. The tumor could not be carried further because of a shortage of C57 black mice.

5) Production of a typical ascites tumor with carcinoma Krebs 2 has already been reported previously (24). Fig. 3 shows approximative median sur-

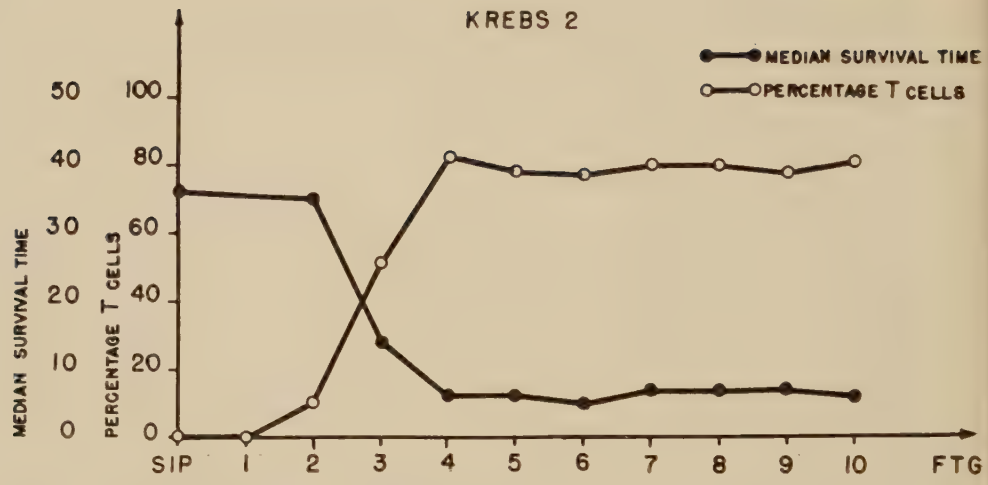


Fig. 3. Relationship between approximative median survival time and percentage of free T cells in the ascitic fluid on 10 FTGs of carcinoma Krebs 2. As in the first three FTGs not pooled exudates were used for further inoculation, but exudates containing the highest percentage of T cells, the T cell frequency of these exudates is shown in the corresponding points. After FTG 4, average T cell frequencies on median survival time ± 2 days are given.

vival times and T cell frequencies during the first 10 transplant generations. In this experiment, 0.3–0.5 ml amounts of exudate were used for inoculation. Dissimilarly to the other tumors reported here, groups of 2 to 10 mice were used, according to the amount of fluid available. Another difference was that upon inoculation of the exudates not several exudates were pooled, but the particular one or two fluids which showed highest concentration of T cells, were further injected until FTG 4. After FTG 4, when the ascites tumor was already well established no such selection was made, but the fluid was routinely transferred in 0.4 ml amounts. Fig. 3 shows a striking decrease in median survival time and increase in T cell frequency during the first 5 FTGs. A difference may be noted as compared with the behaviour of other tumors tested in that upon first inoculation of the solid tumor masses (SIP) median survival time is about as great as on FTG 1, while with most other tumors tested it is much lower. This may be due to the fact, that on the first i.p. transplant of this tumor, which was the first tumor so tested by us, smaller amounts of tumor pulp were inoculated than in later experiments. Other data on this tumor can be found in an earlier paper (24). Morphological appearance of the T cells is shown in Figs. 29–30.

6) The Ehrlich ascites tumor has been investigated previously by the author and the literature has been reviewed (22).

In the present work, some additional experiments were carried out regarding the frequency of T cells and non tumorous exudate cells (pages 527–528), the changes in the composition of the peritoneal fluid after injection of a certain number of T cells (page 542), and relationships between solid infiltration and growth in the fluid (page 550). Some photomicrographs of the tumor resp. ascites cells are shown for comparison in Figs. 26–28.

7) Injection of solid masses from tumor C954 into the peritoneal cavity of C57 leaden mice gave rise to development of intensely hemorrhagic exudates, accompanied by extensive carcinomatosis of the peritoneal cavity. In this hemorrhagic exudates a small number of T cells were detected, which sometimes appeared in small groups. These cells represented 4 per cent of all the cells present, on an average. Median survival time was 12 days. So far only 6 FTG-s have been observed, in which median survival times varied between 20 and 35 days and T cell frequencies up to 25–30 per cent could be observed on the three latest generations. The production of the always very hemorrhagic exudate appeared with great regularity in all generations and in 100 per cent of the animals injected.

8) Only solid tumor and no exudate formation was obtained after inoculation of hepatoma 98/15. The animals died in 32–47 days.

9) Tumor SPAD produced besides solid tumor formation, a hemorrhagic exudate upon intraperitoneal inoculation in 9 out of 10 cases. No T cells could be detected in the exudates. Median survival time (MST) was 35 days. Three FTG-s have been performed so far. MST during them was 58, 65 and 55 days, respectively. No T cells were detected in FTG 1 and 2, while a few T cells appeared in FTG 3, their frequency averaging 1.2 per cent.

10) Tumor SPAH behaved very similarly to SPAD. Hemorrhagic exudates and solid tumors were produced in all 10 animals inoculated. No T cells were detected in the exudates. Median survival time was 20 days on the inoculation of the solid masses, and 56, 72 and 65 days respectively, in the following three FTGs. In all FTGs a low number of T cells could be observed, which, in FTG3, averaged 2 per cent.

11) Tumor SPC3H grew readily in the peritoneal cavity without the production of an exudate, however. The approximative median survival time (MST) was 32 days. One of the developed intraperitoneal tumors was then used again for further i.p. inoculation. In this second group about half of the animals also developed, besides solid tumors, hemorrhagic exudates with no detectable T cells. MST was 35 days. 3 FTGs were produced so far by transmitting the exudate, with MSTs 83, 76 and 90 days respectively. During these generations no T cells could be detected in the smears examined.

12) Besides solid intraperitoneal tumors, tumor SPdba also produced hemorrhagic exudates in 4 out of the 6 animals inoculated. MST was 35 days, no T cells were detected in the exudates. Animals inoculated with the exudates survived an observation period of 6 months, without developing tumors or ascites.

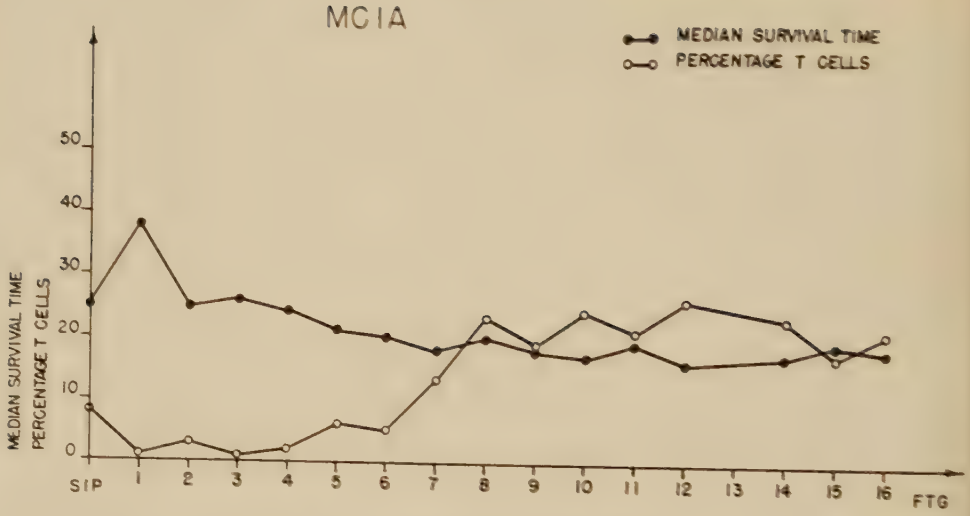


Fig. 4. Same as Fig. 2 in the case of rhabdomyosarcoma MC1A.

b) Sarcomas.

13) Rhabdomyosarcoma MC1A produced a hemorrhagic exudate besides solid tumors in 8 out of 10 animals inoculated, the rest developing only solid tumor. Free T cells were detectable in the fluids with an average frequency of 8 per cent. The approximative median survival times and average T cell frequencies during 16 FTGs performed to date are shown in Fig. 4. A very gradual decrease in survival times and a similarly rather slow and smooth increase in T cell frequencies can be noted. Animals developing only solid tumors and no exudates disappeared entirely since FTG 4. Since about FTG 5 there was an apparent decrease in the amount of grossly observable solid infiltration in the peritoneal cavity and in some animals of the latest generations it could be detected only microscopically. The free tumor cells in the fluid were very large, in general larger than the Ehrlich ascites tumor cells, with hyperchromatic nuclei and often several huge nucleoli. (Figs. 31–34.) Multinucleate cells and normal and abnormal mitotic figures were common. Occasionally giant cells with many nuclei were seen, similar to the typical rhabdomyosarcoma giant cells of the solid tumor (Fig. 34). Average T cell frequencies on the last 5 FTGs were between 19.2 and 25.8 per cent. The frequency of mitotic figures per T cell was found to be 1.25 per cent. The total cell number per ml was 9.5×10^6.

14) Fibrosarcoma MC1E produced only solid tumors, on its first intraperitoneal transplantation, MST being 8.5 days. A second trial was then made, using still larger amount of tumor masses for inoculation. Extensive carcinomatosis was produced with a hemorrhagic exudate in 17 out of 20 mice. The rest had no exudate, only solid tumors. Very few (less than 1 per cent) T cells could be detected in the exudates. Using the exudate, 8 FTGs were performed. T cells were detected in low numbers during all generations, up to a frequency of 15 per cent on generation 6–7. Median survival times were ranging between 17–21 days on all FTGs, not showing any tendency to decrease. Concerning T cell frequency, the variability between individual fluids and generations was too great to establish whether a real increase was taking place. Solid infiltration was always present.

15) Sarcoma MC1M produced on its first intraperitoneal inoculation, solid tumors and heavily hemorrhagic exudates in all animals inoculated. Median survival time (MST) was 13 days. No T cells were detected in the smears of the exudates examined. 19 FTGs were performed. Unfortunately, because of a shortage in C3H mice, it was impossible to carry out consistently the two-group system on each FTG, as carried out with other tumors where one group was used for approximative MST determination, and the other for sacrifice around MST. Only one group was carried on, consisting of 5 mice per FTG. The mice were sacrificed when they showed marked abdominal distension, the fluids collected, counted, and further implanted. MST could thus not be judged for each generation in a reliable way. Fig. 5 shows T cell frequencies in all FTGs. Though these cell frequencies do not occur with certainty at MST, as in the other curves showed, a tendency towards increasing frequency on later generations becomes still obvious. T cells appeared early, namely already in FTG 1. After an initial increase on FTG 2 and 3, their number decreased to rise again after FTG 8 and stayed near 50 per cent after this. The gross appearance of the ascitic fluids changed markedly during the FTGs. In the earliest generations it was very hemorrhagic in all cases, often resembling pure blood. Later on, hemorrhage appeared more irregularly and was less heavy, sometimes the fluid was entirely free from gross blood, and then it often showed an icteric character. The amount of solid infiltration, mainly the grossly observable tumors, decreased markedly in later FTGs, and after FTG 10 they were often hard to find macroscopically. On FTG 16, the following composition of ascitic fluids was found (mean from 4 samples, 600 cells counted in each), 19.8 mononucleate T cells, 3.8 bi- or multinucleate T cells, 11.2 polymorphonuclear leukocytes, 11 lymphocytes, 23 histiocytes + mesothelial cells. Total cell number was 24.8×10^6. The frequency of mitotic figures as percentage

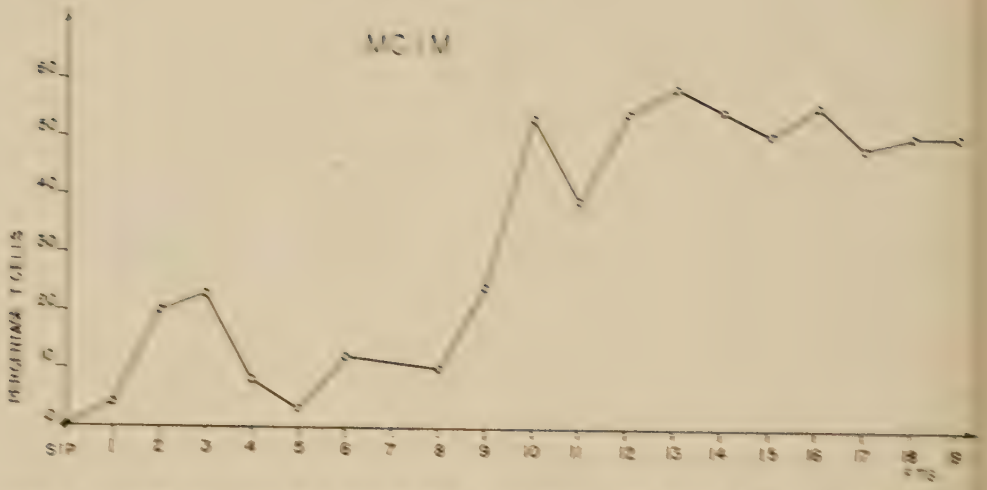


Fig. 3. Average frequency of T cells in ascitic fluids (solid line) and of sarcoma MC1M in the FTGs. SIP: intraperitoneal inoculation of the solid tumor.

of T cells was 2.5 per cent. The fluids counted were collected 14–19 days after inoculation. The T cells were large and hyperchromatic, often with very large nucleoli. Bi- and multinucleate cells were frequent (Figs. 33, 38).

16) Only solid tumor and no exudate formation was obtained after inoculation of sarcoma MC2N. Survival times ranged from 43 to 70 days.

17) T241 gave hemorrhagic exudates in all animals inoculated. Few T cells were detected in these first exudates, their frequency being below 1 per cent. Median survival time (MST) was 9 days. 8 FTGs were carried through, during which no change could be observed in MSTs, which in FTG 1 was 23 days, and in following generations varied irregularly between 20 and 26 days. Hemorrhagic exudates and huge tumors were produced in most animals. In some, however, only solid tumors were produced. T cells were detected in low frequencies, in the 8th generation their frequency ranged between 0.6 and 8 per cent. The tumor was lost after FTG 8 because of a shortage in C57 black mice.

18) Sarcoma 37 produced no accumulation of ascitic fluid on its first intraperitoneal inoculation into 10 Swiss mice but only solid tumors in all animals. MST was 12 days. This experiment was repeated after the author received a copy of the manuscript by Goldie and Felix (1.3) on the growth characteristics of free Sarcoma 37 cells in the peritoneal fluid during a visit to the National Cancer Institute in November 1956. On this

second trial one out of 10 animals showed the accumulation of a hemorrhagic exudate on the 7th day after inoculation. The rest of the animals showed only solid tumors resp. infiltration, and had an MST of 9 days. The hemorrhagic exudate appearing in one animal was collected. Surprisingly, and in contrast to all other carcinomas or sarcomas examined, 40 per cent T cells were found in this fluid on this first implantation of the solid. 2.5 per cent of the T cells were in mitosis. There was a marked leukocytic reaction, in agreement with the findings of Goldie and Felix (13). Over 14 FTGs were performed thereafter, in white stock mice, but the growth was also tested in groups of 5 5 C57 black, C57 leaden and dba mice. MSTs and T cell frequencies showed very irregular behaviour and great variability between different animals in all generations. In some animals up to 70 per cent T cells were counted. (Figs. 39–41.) These were the highest concentrations found. In other animals there was a marked leukocytic reaction and low T cell frequencies. Sometimes T cells were not detectable at all. Only solid tumor formations without exudate production and spontaneous regressions were common. MSTs varied in animals inoculated with 0.4 cc exudate between 6 and 15 days, the variation not being correlated to FTG number. In order to test whether this variation was not due to great differences in injected T cells in different FTGs, 3 groups each consisting of 10 mice were injected with 1.16×10^6 T cells, originating from different donors. MSTs in these three groups were 15, 18 and 25 days, respectively. Great variations were also present between different mice of the same inbred strains, spontaneous regression, low and high T cell frequency all being present in the C57 black group. In the C57 leaden mice there was spontaneous regression in 2 out of 5 cases, only solid tumor formation in 2 cases, and ascites formation with 25 per cent T cells in one case. In the exudates with high T cell frequencies necrotic cells were often present, which agrees with the findings of Goldie and Felix (13).

19) Sarcoma 180 produced only solid tumor formation with no exudate in any of the animals. The animals died in 10–17 days.

20) The Wagner osteogenic sarcoma took in only 6 of 10 animals inoculated. In 4 animals only solid tumors were present, while two developed a white albuminoid exudate. These exudates were injected into 5 mice. However, the animals injected survived an observation period of 2 months without tumor development.

21) The Yoshida rat ascites sarcoma was found to take and kill in about 80 % of non-inbred albino rats. This tumor has been investigated in detail by Yoshida (51). The tumor was used for nucleic acid determinations (page 547).

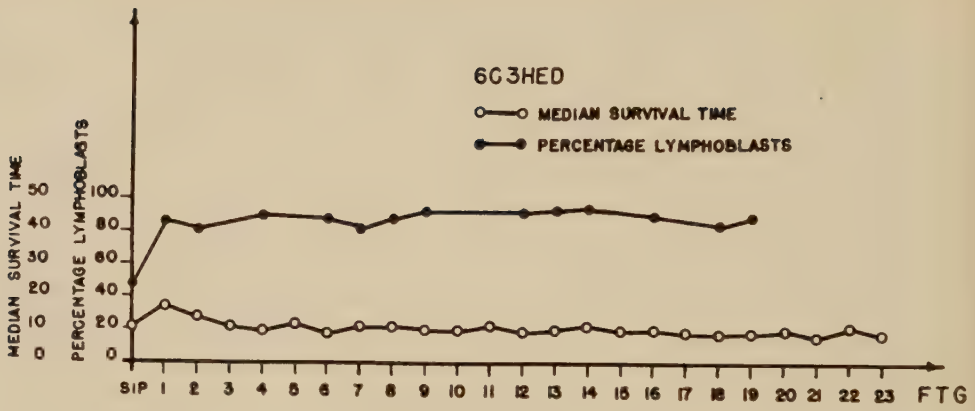

6C3HED
MEDIAN SURVIVAL TIME
PERCENTAGE LYMPHOBLASTS
MEDIAN SURVIVAL TIME
PERCENTAGE LYMPHOBLASTS
SIP 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 FTG

Fig. 6. Same as Fig. 2 in the case of lymphoma 6C3HED.

c) Lymphomas.

22) Lymphoma 6C3HED produced, after its first i.p. inoculation, ascites development in all animals. The animals died in 9–11 days. All ascitic fluids contained lymphoma cells. As mentioned on page 529 the differential counting of lymphoma cells in the peritoneal fluid is much more difficult than that of the huge carcinoma and sarcoma cells of the other tumors tested, mainly because of the small size of the cells. Thus the percentages of lymphoma cells can only be approximative. With this reservation in mind, the following findings were made: after the first injection of the solid tumor into the peritoneal cavity, the ascitic fluids developing contained mainly two elements around MST: lymphoma cells with an average concentration of 48 per cent and necrotic cells with pyknotic and karyorrhectic nuclei with an average concentration of 39 per cent. Frequency of mitotic figures was about 1 per cent of the intact lymphoma cells. 23 FTGs were then performed. MSTs were found to be extraordinary regular in all generations as shown in Fig. 6. After FTG 3 their range was 8–12 days in the next 20 generations, which is a remarkable constancy when compared with the carcinomas and sarcomas tested. The range of individual survivals was 7–16 days. Differential cell counts were not carried out on each generation, but it is still clear that the curve denoting the frequency of the cells counted as lymphoma cells is fairly horizontal and the variations on different generations are rather small. These variations were not so much due to variations in the frequency of cells counted as normal or inflammatory peritoneal cells, but much more to differences in the frequency of pyknotic cells. Pyknotic cells were always present, but

their number has not been as high as on the first injection of the solid, if the fluids were collected around MST. Animals surviving more than 2 days after MST had a higher frequency of necrotic cells than those examined around MST.

The total number of lymphoma cells on MST per ml was counted in 10 cases. It ranged between 86–181 $\times 10^6$ cells/ml, 144 $\times 10^6$ cells on an average. The frequency of mitotic figures as a percentage of morphologically intact lymphoma cells ranged between 0.3–1.6 per cent.

The appearance of 6C3HED cells is shown in Figs. 42–43. Peritoneal infiltration was always present. It was most conspicuous in the region of the pancreas but was to be found all over the mesentery and fatty tissue.

23) The lymphoma in dba mice produced ascites development in all animals after its first i.p. inoculation. The animals died in 8–13 days. All ascitic fluids contained lymphoma cells in high concentrations; their average frequency being 73 per cent. There was a number of necrotic cells present with nuclei showing pyknosis and karyorrhexis, similarly to 6C3HED. Average frequency of necrotic cells was 21.5 per cent on median survival time $\pm$ 2 days. The mitotic index was 0.75 per cent on an average. 18 FTGs were then performed. In general, the behaviour of this tumor was very similar to that of the previous one (6C3HED). The approximative median survival times were in the same range on all FTGs, not showing any correlation with the FTG number. However, MSTs were less constant and the variations greater, than with 6C3HED. There was a range between 11 and 17 days. The individual survivals ranged between 8 and 28 days, under the standard conditions used (0.4 ml exudate injected which was collected around median survival time). The frequency of lymphoma cells was generally high. It varied between 75 and 94 per cent (85.7 per cent on an average), not being correlated to the FTG number. These figures refer to conditions on median survival time. Total number of lymphoma cells varied between 71.9–319.8 $\times 10^6$ cells per ml (167.5 $\times 10^6$ average). Frequency of necrotic cells was highest in the first generation, after i.p. injection of the solid masses, but necrotic cells were always present even in later generations. Median survival times of groups inoculated with counted numbers of lymphoma cells were as follows: 10 million cells: 16 days, 3.6 million cells: 19 days.

The cells of dba lymphoma were similar to those of 6C3HED (Figs. 44–45). The appearance and extension of peritoneal infiltration was also very similar.

24) The Patterson lymphosarcoma behaved similarly to the previous

two tumors in that it produced on its first i.p. inoculation an ascites formation with high concentration of lymphoma cells in 6 out of 10 mice, namely 80 per cent on an average. In the rest of animals the tumor failed to take. Average frequency of necrotic cells was 3.5 per cent, the rest of the cells being different normal constituents of the peritoneal fluid. Survival times varied between 5 and 9 days. Only four FTGs were carried through, then the tumor was discarded because of the low (about 50 per cent) frequency of takes. In these four generations there was a rather constant high concentration of lymphoma cells—up to 97 per cent – in all the animals affected, which survived between 5 and 10 days. Morphologically the lymphoma cells were very similar to both previous tumors, as shown in Figs. 46–47. Peritoneal infiltration was always present, similar in character to the two previous lymphomas.

25) The Krebs-Rask Nielsen-Wagner lymphosarcoma has been tried in some experiments because Krebs and his collaborators reported (26) that after 10–12 days following on intraperitoneal injection of the solid tumor they obtained an ascites containing the lymphoblast-like tumor cells, which was capable of further transmitting the tumor. These findings could be completely confirmed. Injection of the solid tumor produced an ascites with over 90 per cent average concentration of lymphoma cells. MST was 11 days. After the injection of 0.4 ml exudate through 8 FTGs MST varied between 10 and 13 days. Ascitic fluids were similar over all generations. The variability between the composition of the different peritoneal fluids was low and was caused more by a varying number of necrotic cells than by inflammatory cells, the frequency of which was low.

At the end of the survey of the four lymphomas tested, it may be worth while to point out the following *common features* in all four: 1) The first injection of the solid tumor already caused an ascites formation with a very high frequency of lymphoma cells, 2) With the exception of dba lymphoma which sometimes was slightly pink, the ascites fluids were never grossly bloody; a marked difference against all other tumors tested, where the amount of blood varied enormously from fluid to fluid and where intensely bloody fluids always reappeared, 3) Concentration of lymphoma cells and median survival time was in general independent of FTG number, 4) Inflammatory cells were rare and occurred in low frequency, 5) There were always some and often many necrotic cells present, with pyknotic and karyorrhectic nuclei, 6) Survival times were fairly similar and varied within comparatively narrow limits.

d) Other tumors.

26) C 1300 produced huge solid tumors in all animals after i.p. inoculation. 0.5–2 ml amounts of bloody ascites were present in about half of the animals. T cells were detectable in these bloody fluids, their average frequency being 1.2 per cent. Median survival time was 20 days. 7 FTGs were performed. During all these generations there was no change in the marked tendency towards solid tumor formation, and in each generation about half of the animals had no ascites but only solid tumors. T cell frequency was 6 per cent in FTG2, 12 per cent in FTG4, and between 25 and 31 per cent in FTGs 5–6 and 7 thus showing a certain tendency to increase. T cells were often present in small complexes. The rest of the cells present consisted only to a small degree of well preserved inflammatory or normal cells, and to a great deal of cells with pyknotic nuclei which did not permit their differential diagnosis. Inflammatory reaction was always very small or absent and the inoculations took in 100 per cent. Median survival times varied over a comparatively wide range, namely between 18 and 29 days, 23.9 days on average. The T cells appeared often in small complexes. They were moderately hyperchromatic and often large in size, but there was a marked variability. (Figs. 48–49.)

27) Injection of the Harding Passey melanoma produced only solid tumors after a latency period of 3–6 months. In three instances, animals were laparatomized, the abdominal tumors opened, minced and widely distributed over the peritoneal cavity. Puncture of the peritoneal cavity 4 days after the operation showed a somewhat increased amount of peritoneal fluid (0.3–0.5 ml) and many of the macrophages in the fluid were found to contain large amounts of melanin granules. The presence of T cells could not be ascertained, however. After 8 days the amount of the peritoneal fluid was as usual and there were only a very few macrophages left with melanin granules.

28) Injection of melanoma S 91 into dba mice resulted in a hemorrhagic ascites formation after 44 days in one out of 10 animals. The rest developed solid tumors after 2–5 months. 5 animals injected with the hemorrhagic ascites (in which no T cells could be detected) survived an observation period of 7 months without tumor or ascites development.

V. *Viability tests*

To get some orientation on the frequency of dead T cells in different fluids on median survival time, Schrek's test (44) was carried out in a number of cases. Fluids with over 50 per cent T cell frequency were always chosen and 500–1 000 cells were counted. The results are given in Table I.

It is to be noted that in all cases tested the percentage of dead cells is rather low. This applies however only to median survival time. Preliminary experiments with different tumors have shown that the percentage of dead cells can become higher in animals which live considerably longer than median survival time.

It applies also only to tumors that have passed a number of FTGs and not to fluids that develop after intraperitoneal inoculation of solid tumors.

VI. *Subcutaneous reinoculation* of ascites fluids which was performed with all tumors after FTG 3, produced solid tumors regularly, as could be

Table I

Percentage of Dead Cells around Median Survival Time.

Tumor	No. of samples	Percentage of dead cells		Average per cent T cells
		Range	Mean	
Ehrlich	12	0.2— 4.5	1.8	83.4
Krebs 2	8	0.6— 6.4	2.3	81.7
15091 a	7	2.4—13.0	5.3	68.4
MC1M	7	0.1— 6.6	2.1	57.4
6C3HED	8	0.8— 5.2	1.6	94.2
dba lymphoma	12	0.6— 9.2	2.9	92.6

expected. Comparison between the histological structure of these solid tumors and the original tumors which have been used for the first intraperitoneal transplantation failed to reveal any obvious change in either the structure of the tumor, or the morphology of the cells. Thus, for instance, the typical giant cells of rhabdomyosarcoma MC1A could be detected even after the tumor has passed 16 FTGs. Similarly, the well differentiated spontaneous mammary adenocarcinomas SPAD, SPAH, SPC3H showed their typical structure again after 3 FTGs. The only exceptional tumor was TA3 which, after the 10th FTG, showed some tendency for further anaplasia, but, as mentioned above, this tendency was already marked before we got the tumor and used it for this work.

VII. *Changes in the composition of peritoneal fluid during development of the Ehrlich ascites tumor*

Yoshida (51) and Goldie & Felix (13) have studied the composition of the peritoneal fluid after the injection of Yoshida sarcoma cells or cells of Sarcoma 37 and the lymphoma in dba mice, respectively. It therefore was of interest to study the same happenings with other tumors. Preliminarily, such studies were carried out on the Ehrlich ascites tumor.

Groups of dba × C57 leaden F1 hybrid mice were used for these experiments. 10 mice have been inoculated with about 25×10^6 cells, while a control group of 7 mice received only cell-free ascites serum, which was prepared by centrifugation of the cells, collection of the supernatant and 4 times repeated quick freezing and thawing. Withdrawals of the peritoneal fluid were then carried out at certain intervals, smears were prepared and the occurring cell types were differentiated. Figs. 7, 8

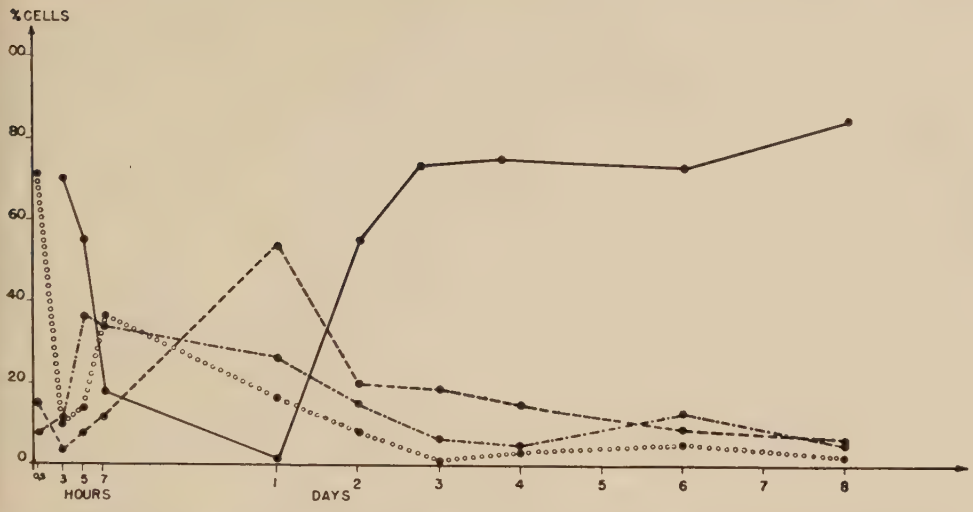


Fig. 7. Changes in the composition of peritoneal fluid in dbaxC57 leaden F1 hybrid mice after the inoculation of 25×10^6 Ehrlich ascites T cells. —— % T cells; - - - - % mesothelial cells + histiocytes; · - · - · - · % polymorphonuclears; o o o o o o % lymphocytes.

and 9 show the results. Fig. 7 shows the changes in cellular composition in the group injected with tumor cells. Fig. 8 refers to the control group. Fig. 9 is from the same data as Fig. 7, however the tumor cells have been entirely omitted and the frequency of other cell elements calculated and plotted in relation to each other, to make possible a direct comparison with Fig. 8. The initial relative increase in polymorphonuclears which occurs in control and also tumor cell injected groups should be noted. In the former, this occurs earlier and its peak is lower. After the cessation of reaction of polymorphonuclears, there is a pronounced relative increase in mononuclears, which occurs earlier and soon drops again in the control group, but stays high in the tumor group. The relative frequency of the tumor cells themselves drops markedly during the first 24 hours. It cannot be decided on the basis of the present data whether this drop can entirely be accounted for by an increase in other cell types or also by a number of tumor cells disappearing from the fluid by disintegration, adhering to the peritoneal surface, etc. This would only be possible if the entire number of tumor cells in the whole peritoneal fluid could be counted after quantitative collection of the peritoneal fluid. After 24 hours, a gradual relative increase in the tumor cells starts and they increase to a certain maximum. The percentage of detectable necrotic cells is negligible during the whole course of this development.

The findings in Fig. 7 are very similar to those reported by Yoshida for his sarcoma both in regard to the character of the changes and the times when they appear. The two early reactions, that of the polymorphonuclears and that of the mononuclears (histiocytes and mesothelial cells) occur even in the control group. Later on, however, the control animals return to a normal mainly lymphocytic

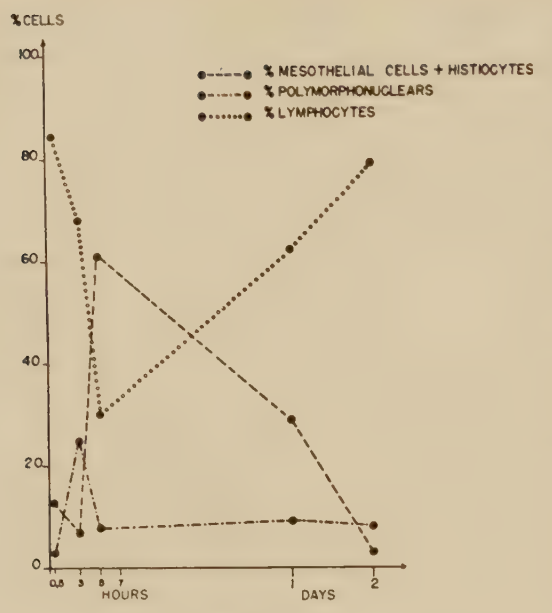


Fig. 8.

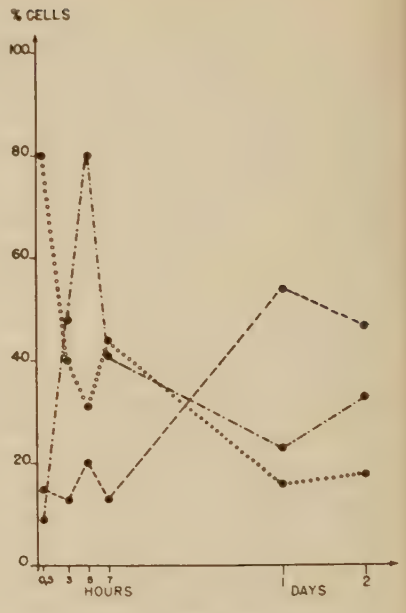


Fig. 9.

Fig. 8. Control group to experimental group in Fig. 7. This group has been inoculated with the same volume of cell-free ascites serum.
Fig. 9. The data presented in Fig. 7 recalculated after omitting the T cells to show relative changes of other cell elements.

peritoneal fluid, while the non-tumorous cells predominating in the tumor fluids are mainly large mononuclears. In agreement with both Yoshida and Goldie & Felix, mast cells have never been observed after the tumor cell multiplication has become marked, in other words after the first day.

Goldie & Felix found that Sarcoma 37 cells elicit a marked leukocytic reaction from the host and after the initial increase of tumor cells an increasing necrosis of the cells follows, corresponding to a final stage of inactivity and partial regression. On the other hand the lymphoma in dba mice examined by them did not show this but showed a gradual increase of neoplastic cells over the whole course of tumor development. This difference was attributed to the strain specific qualities of lymphoma cells for dba mice and the foreign nature of Sarcoma 37 cells for the implanted hosts, respectively. However, the Ehrlich ascites tumor and according to Yoshida, the Yoshida sarcoma, both of which are not strain specific, behaved more similarly to Goldie's lymphoma than to Sarcoma 37. In our experiments Sarcoma 37 showed a rather irregular behaviour compared with other tumors studied, as mentioned previously. It seems to present a rather special case and it is doubtful whether this behaviour can be entirely accounted for by its foreign quality for the mice used.

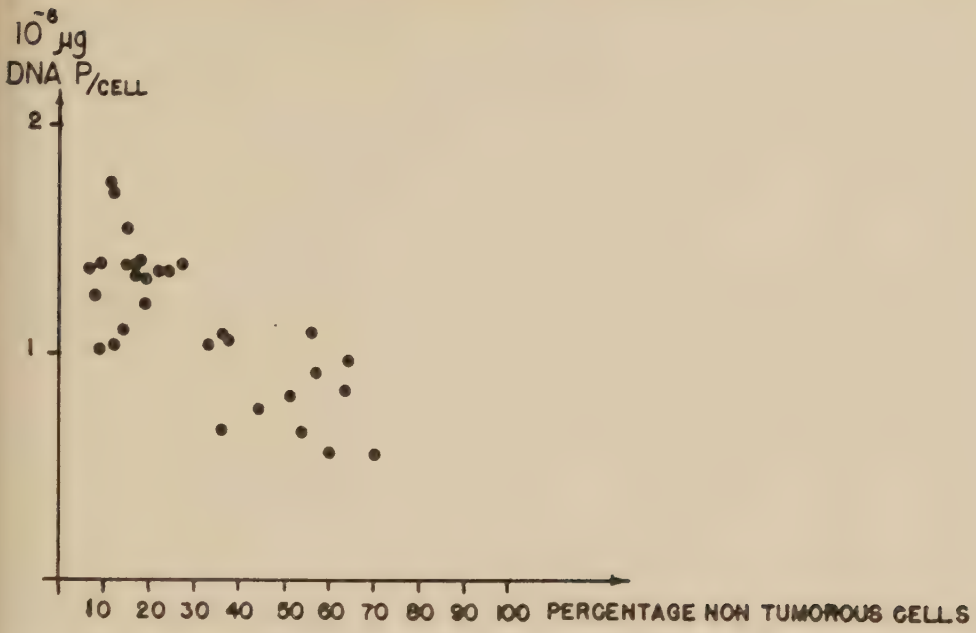


Fig. 10. Effect of different admixture of non tumorous exudate cells on the desoxypentose-nucleic acid content per total cell number of the Ehrlich ascites tumor. The higher frequencies of non tumorous exudate cells were produced by injections of 0.15–0.5 mg doses of acriflavine.

VIII. *Nucleic acid determinations*

Nucleic acid determinations were performed using the same methods as in previous work of Goldberg, Klein & Klein (12) where the errors of the methods used were also established. The only exception was that DNA phosphorus was not calculated as the difference between NA and PNA phosphorus, but was determined separately (43). Methods of cell counting were also identical with those in the previous work.

A prerequisite for these determinations is that all detectable nucleic acid phosphorus shall be intracellular and none in the suspending ascitic fluid. It has been established previously (21) that this is the case with the Ehrlich ascites tumor. As the present determinations also involved other different tumors, one duplicate has been centrifuged and the supernatant decanted, while the other duplicate has been determined in the usual way, using the whole fluid. If any significant amounts of nucleic acid phosphorus were present in the fluid proper, a systematic difference between the two duplicates is to be expected. Such a difference was never detected. A few determinations which have been carried out on cell-free supernatant fluids also gave entirely negative results.

Only such samples were used for nucleic acid determinations where a concentration of T cells of at least 40 per cent was reached. This was established on smears

prepared immediately after collection of the ascitic fluids, whereafter the suitable fluids were selected. Determinations were thus possible on carcinoma 15091a, sarcomas 37, MC1M, lymphomas 6C3HED and the lymphoma in dba mice, and the Yoshida rat sarcoma. The results are shown in Table II. Carcinoma TA3 could not be used in spite of the high T cell concentrations because of the occurrence of cell complexes in the fluids. Determinations on the Ehrlich carcinoma, Krebs 2 carcinoma and Krebs-Rask Nielsen-Wagner lymphoma have been reported previously (12, 20, 24). In the case of the Ehrlich carcinoma, new determinations were carried out as a control for the procedures used. The nucleic acid values obtained agree fairly well with the previous values though they are slightly lower. This can be explained by the somewhat higher concentration of non-tumorous cells in the present material. In the case of the Krebs lymphoma and carcinoma Krebs 2, the previously reported values were used. The fluids were in all cases used around median survival time $\pm$ 2 days and after at least 10 FTGs.

As all samples contain a certain number of non-tumorous cells, the nucleic acid values per total cell number do not directly express the average nucleic acid content of the single tumor cell. It has been shown previously (12) that normal exudate cells contain smaller amounts of both PNA and DNA than cells of the Ehrlich ascites tumor, thus their admixture will result in a too low value when the nucleic acids are calculated per single T cell. For DNA, this is shown in Fig. 10, where the DNA content per ml is plotted against the frequency of non-tumorous exudate cells, the variable admixture of which has been provoked by different doses of acriflavine. (Acriflavine was previously shown (22) to have no effect on DNA content of the cells.) On the other hand, if nucleic acid content per T cells alone will be expressed, it will be too high, since the nucleic acid content of the exudate cells is then neglected and it increases the T cell value. Using both values, they will give the limits between which the average NA content of the single T cell lies. Naturally, the discrepancy between the two values will be higher with increasing frequencies of non-tumorous exudate cells.

The values in Table II permit the following conclusions:

a) If, as previously (22), the range for normal somatic mouse cells found by different authors is taken to lie between $0.5–0.8 \times 10^{5}$ µg DNA per nucleus, all three lymphomas examined show a DNA/cell value which is within this range. The fluids used were all highly uniform and contained high concentrations of the lymphoblast-type cells which are supposed to be identical with the malignant elements of these tumors.

b) The three carcinomas (Ehrlich, Krebs 2, 15091a) and two mouse sarcomas (Sc 37, MC1M) examined all show a significant increase of average DNA per cell values beyond the normal range. The extent of this increase can be judged with exactness only in the case of the Ehrlich and Krebs 2 tumors, where the T cell concentration is very high. In the Ehrlich tumor it is about twice the normal value, in the Krebs 2 it is somewhat less than twice. In the other three the minimal values (values per total cell num-

TABLE II

Nucleic Acid Content of Ascites Cells from Different Tumors.[3]

Tumor	Number of samples	Average number of cells $\times 10^6$ per ml	Average percentage T cells	Of 100 T cells		PNA/ total cell number 10^{-6} µg P[1]	(PNA/ T cell)[4]	DNA/ total cell number 10^{-6} µg P[2]	(DNA/ T cell)[4]	Ratio $\frac{\text{PNA}}{\text{DNA}}$
				multinucleated	mitosis					
15091a ca.	7	49.9	62.0	4.5	1.13	2.98	(5.02)	1.69	(2.63)	1.94
6C3HED lymphoma	7	148.5	95.5	1.4	0.8	0.82		0.61		1.30
dba lymphoma	9	174.6	94.3	0.2	0.4	0.73		0.74		0.97
Sarcoma 37	7	49.5	53.2	0.2	1.1	2.19	(4.77)	1.23	(1.88)	2.06
MC1M sarcoma	14	25.3	49.2	4.4	1.9	3.17	(7.18)	1.40	(2.71)	2.41
Yoshida sarcoma	7	80.8	82.6	0.2	0.85	1.25	(1.51)	0.88	(1.06)	1.45
Krebs 2 ca. (ref. 24)	14	142.0	83.0	0.7	2.5	2.16	(2.60)	1.14	(1.37)	1.81
Krebs lymphoma (ref. 20)	15	246.1	92.0			0.58		0.70		0.83
Ehrlich ca.	13	135.0	81.8	0.9	2.8	2.37	(2.92)	1.31	(1.64)	1.97
Non tumorous exudate cells (ref. 12)	15					0.44		0.77		0.59

[1] Corresponds to about 10.6 times more PNA.
[2] Corresponds to about 10.1 times more DNA.
[3] The errors of the methods used were established in ref. 12.
[4] Not calculated in cases of T cell frequencies higher than 90 per cent. The average PNA and DNA content of the single T cell lies between the value calculated per total cell number and that calculated per T cell (see text).

ber) are increased about twice, thus the real value must be still higher. In the Yoshida rat sarcoma, there is a slight increase of DNA/cell, which, however, is not significant.

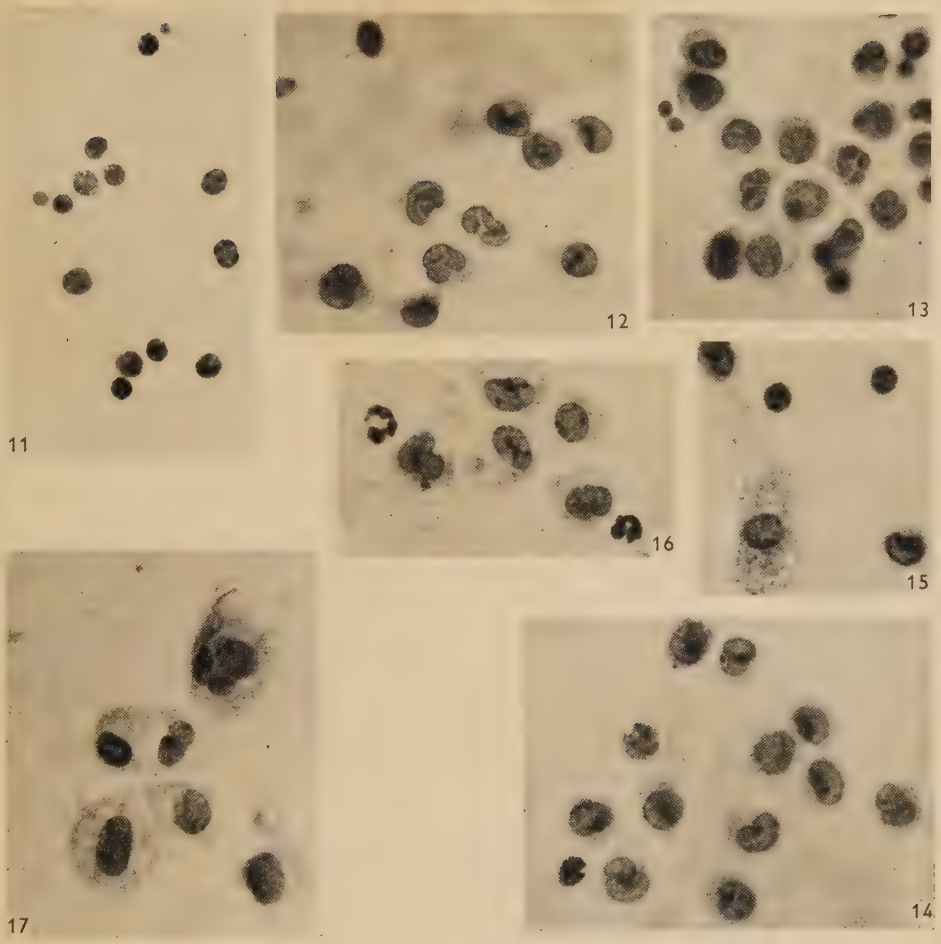
c) In relation to the DNA values, the PNA contents are relatively lower in the tumors with low DNA content per cell (lymphomas) than in those with increased DNA (carcinomas and mouse sarcomas). The PNA/DNA ratios range in the former cases between 0.83–1.30, in the latter cases between 1.81–2.41. This latter in spite of the relatively higher admixture of exudate cells, which lowers the PNA/DNA ratio. Yoshida's rat sarcoma has an intermediate position.

d) There is no certain correlation between the increase in DNA or PNA and the mitotic index with the exception that the lymphomas have somewhat lower mitotic index than the tumors with high DNA. This may however not be significant due to the greater difficulty in counting late prophases and early metaphases in the small lymphoblast cells with dense chromatin.

IX. *Ultraviolet microphotograpy*

Although single photomicrographs in monochromatic ultraviolet light cannot be used for any quantitative judgment as to the amount of nucleotides present in the cells, the presentation of some of such pictures can still be of some interest, with regard to the especially high absorption at 257 mμ of the nucleotides, and give some information as to the variability in absorption of different cells. Another reason for taking such photomicrographs was the possibility of obtaining detailed pictures of the living cell, where also a good orientation about cell size can be made. In the case of the spherical exudate cells, better pictures could be obtained of the living cell than with other methods, e.g. phase microscopy.

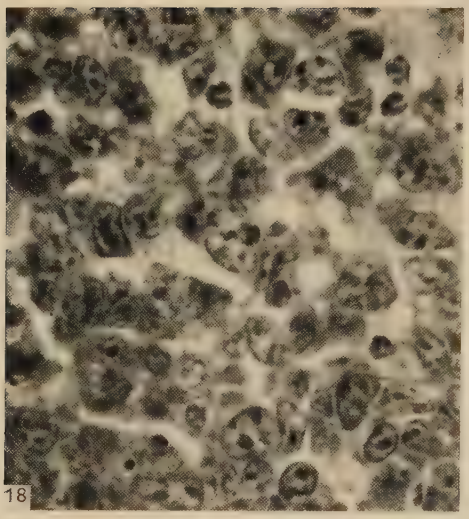
Ultraviolet photomicrographs were taken in the Köhler U.V.-microscope at 257 mμ. Three types of samples were selected: The Ehrlich ascites tumor with its high DNA and PNA content; the lymphoma in dba mice with its non elevated DNA and relatively low PNA, and, for comparison, normal exudate cells from the peritoneal fluid of a C57 leaden mouse. Fresh drops from all exudates have been put on quartz slides immediately after the fluid has been withdrawn. A quartz coverslip was put on and the preparation was surrounded with paraffin. Photomicrographs were immediately taken by focusing on one part of the preparation and then moving the microscope stage to a non-irradiated part of the slide where the plate was exposed. Thus the cells photographed were only exposed to U.V. light during the 10 seconds of exposure. Plates have been exposed, developed and printed under strictly identical conditions.

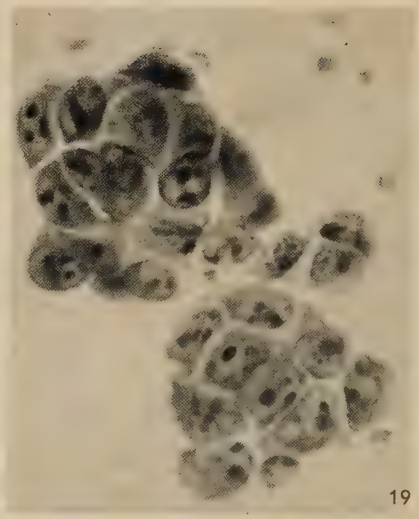


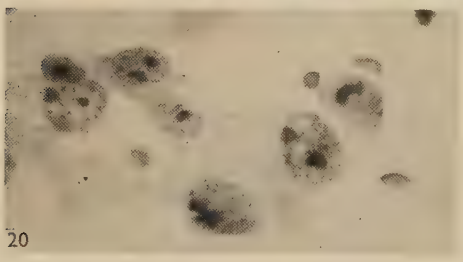
Figs. 11–49 show smear preparations of peritoneal fluids and sections of some solid tumors, respectively. All smears have been prepared according to the method of Papanicolaou. The sections were cut of formalin fixed, paraffin embedded material at 5 μ thickness and stained with heamatoxylin-eosin. Magnification: 700 ×.

Fig. 11. Normal peritoneal fluid of a C57 black mouse, containing mainly lymphocytes.
Figs. 12–15. Normal peritoneal fluids from dba mice. Note mesothelial cells and histiocytes. One mast cell in Fig. 15.
Figs. 16–17. Peritoneal fluid 6 days after Kieselguhr injection. Mainly mesothelial cells present with some polymorphonuclears.

Figs. 50-52 show a larger cell size and a somewhat higher absorption in the case of the Ehrlich ascites cells than in the case of cells from dba lymphoma or of the normal exudate. The variations between different cells







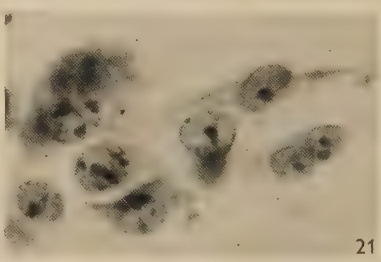


Fig. 18. Field of solid TA3 carcinoma.
Figs. 19–21. Small T cell complexes from TA3 peritoneal exudates after 9 FTGs. Note large nucleoli.

in different cases will be further investigated with the help of quantitative measurements.

Fig. 53 has been prepared from isolated nuclei of the Ehrlich ascites tumor, similarly at 257 mμ. Such nuclei are very easily prepared from this material, as shown previously (12). Though the photographic conditions are not identical with those in Figs 50–52, the picture is showing the nuclear details which are overshadowed by the intense cytoplasmic absorption in Fig. 50.

X. *Relationships between solid growth on the peritoneum and ascites tumor*

In the case of the Ehrlich and Krebs 2 ascites tumors, these relationships have been investigated to some extent in a previous work (24). There an inverse relationship was found between the number of inoculated cells and the extent and amount of solid infiltrative growth on the peritoneum around median survival time.

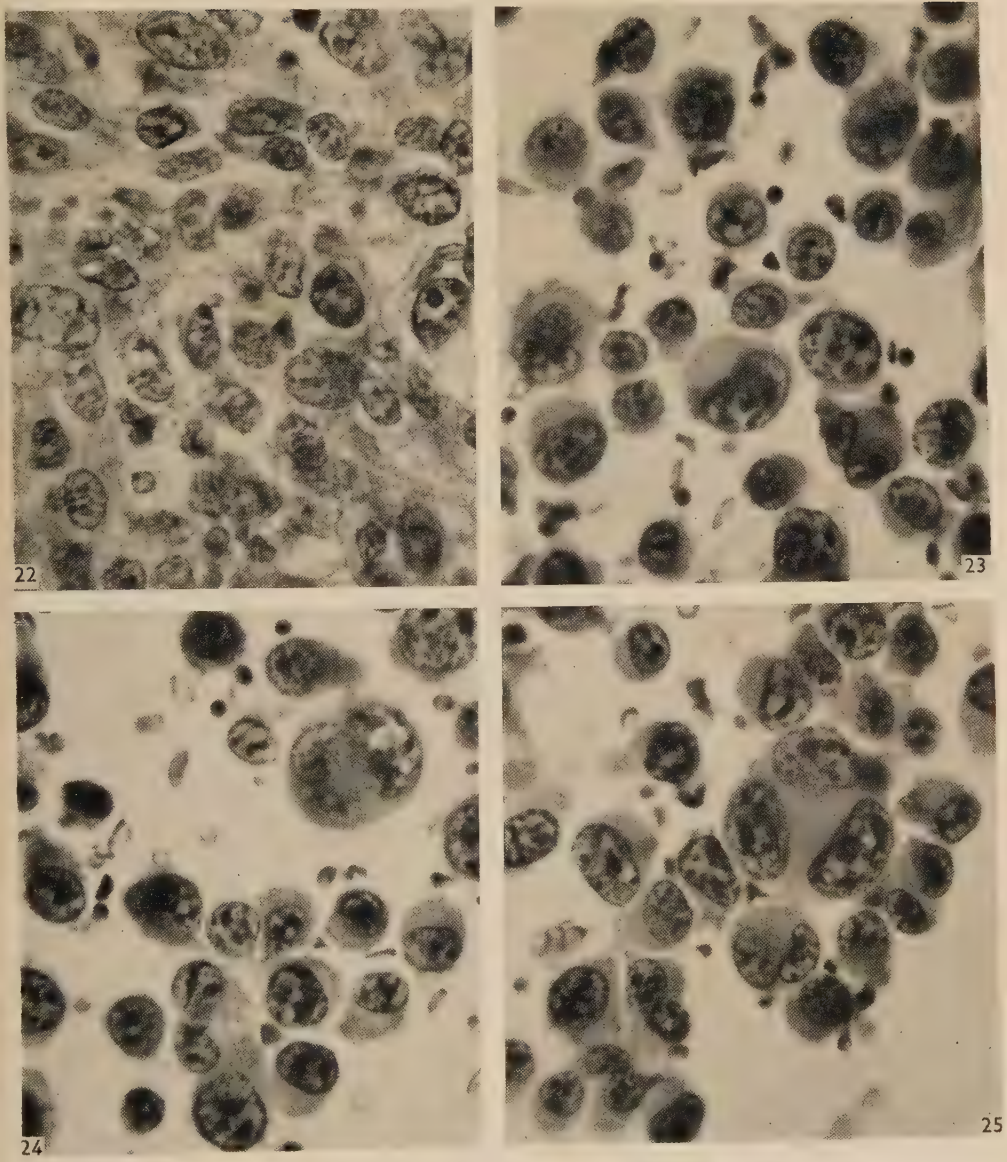
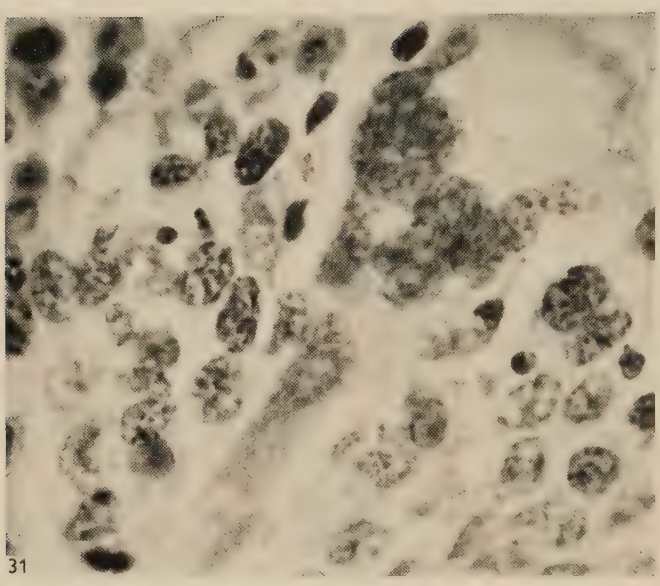
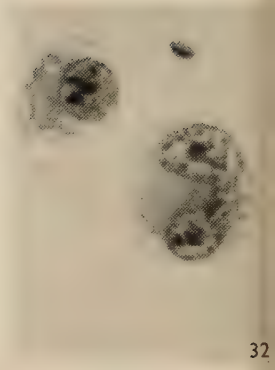


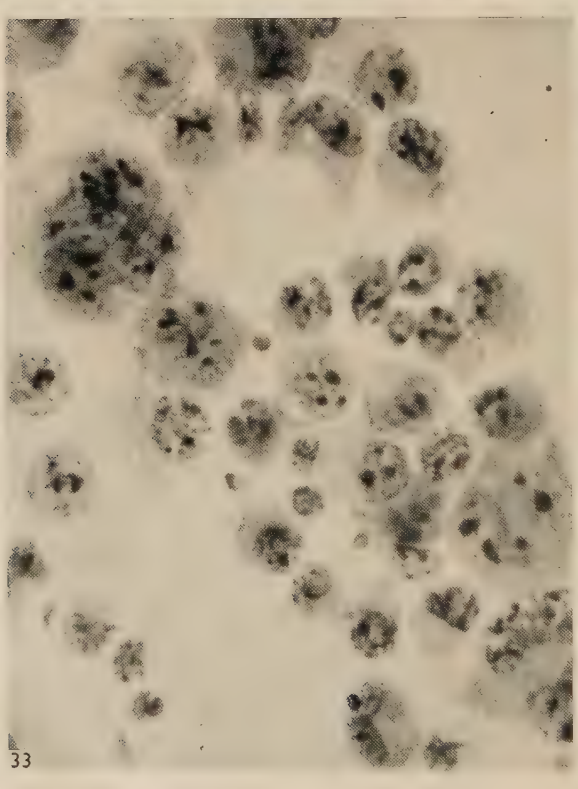
Fig. 22. Field from solid 15091a carcinoma.
Fig. 23–25. Peritoneal exudates of 15091a carcinoma after 9–11 FTGs. Note hyperchromatic T cells with large nucleoli, big variations in size and occurrence of bi- and multinucleated cells. All fluids contain a number of erythrocytes.



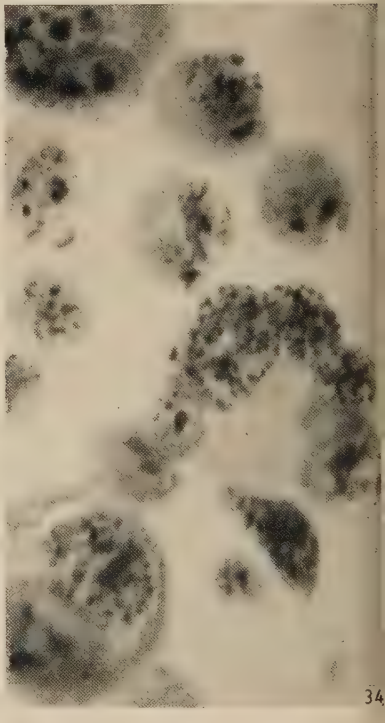
31



32



33



34

from the normal towards neoplastic cells in serous effusions "takes the direction of irregularity in size and particularly increased size of the cell, enlargement and hyperchromatism of nucleus, increased prominence of nucleolus." Ivers and Pomerat (17) state that despite the great multitude of different cell types often encountered, it is often easy in neoplastic exudates to demonstrate the presence of cells that definitely do not resemble in any way the elements seen in the fluid in non-neoplastic conditions. Saphir (42), using the method of Papanicolaou, states that this method is superior to the embedding methods used by many previous authors. He describes mesothelial and malignant cells in detail and gives directions as to their differential diagnosis. He points out among others that mesothelial cells have a clear cut and entirely round nucleus, and a small nucleolus. Malignant cells are stated to have large, irregular nuclei frequently with big nucleoli and large amounts of cytoplasm. Normal mitoses occur also in mesothelial cells but atypical mitoses are characteristic of tumor cells.

Following the method of Papanicolaou, the members of the Vincent Memorial Laboratory Staff (48) name among others following characteristics by which a differentiation of malignant from normal cells is possible: "increase in nuclear content, irregular arrangement of the chromatin, large nucleoli and variation in size and shape."

In the case of the fully developed ascites tumor we are dealing with a condition considerably different from human neoplastic exudates. This condition is reached with a number of tumors only after a number of repeated transfers always using the exudate, and the capability of the exudate to induce tumor growth on the peritoneum is at the same time a proof that it contains tumor cells. This capability, as judged by the number of takes, the time of tumor development and survival time, increased definitely during the subsequent transfers with most carcinomas and sarcomas tested. Closely parallel to this increase there was another increase going on: an increase in the relative and absolute frequency of the cells diagnosed as T cells. In the case of the carcinomas and sarcomas tested, these cells are entirely foreign to the normal or inflammatory exudate, and borderline cases rarely occur. In the case of the Ehrlich, Krebs 2, 15091a carcinomas and the MC1A, MC1M and Sc37 sarcomas, where it was possible to reach a sufficiently high concentration of T cells, the great number of these cells

---

Fig. 31. Field of solid MC1A rhabdomyosarcoma. Note typical giant cell.

Figs. 32–34. Free T cells from MC1A peritoneal exudates on 13–14th FTG. Note huge nucleoli, multinucleated cells and marked variations in size. Giant cell in Fig. 34 is similar to some giant cells in the solid tumor. Several histiocytes are present in Figs. 33 and 34.

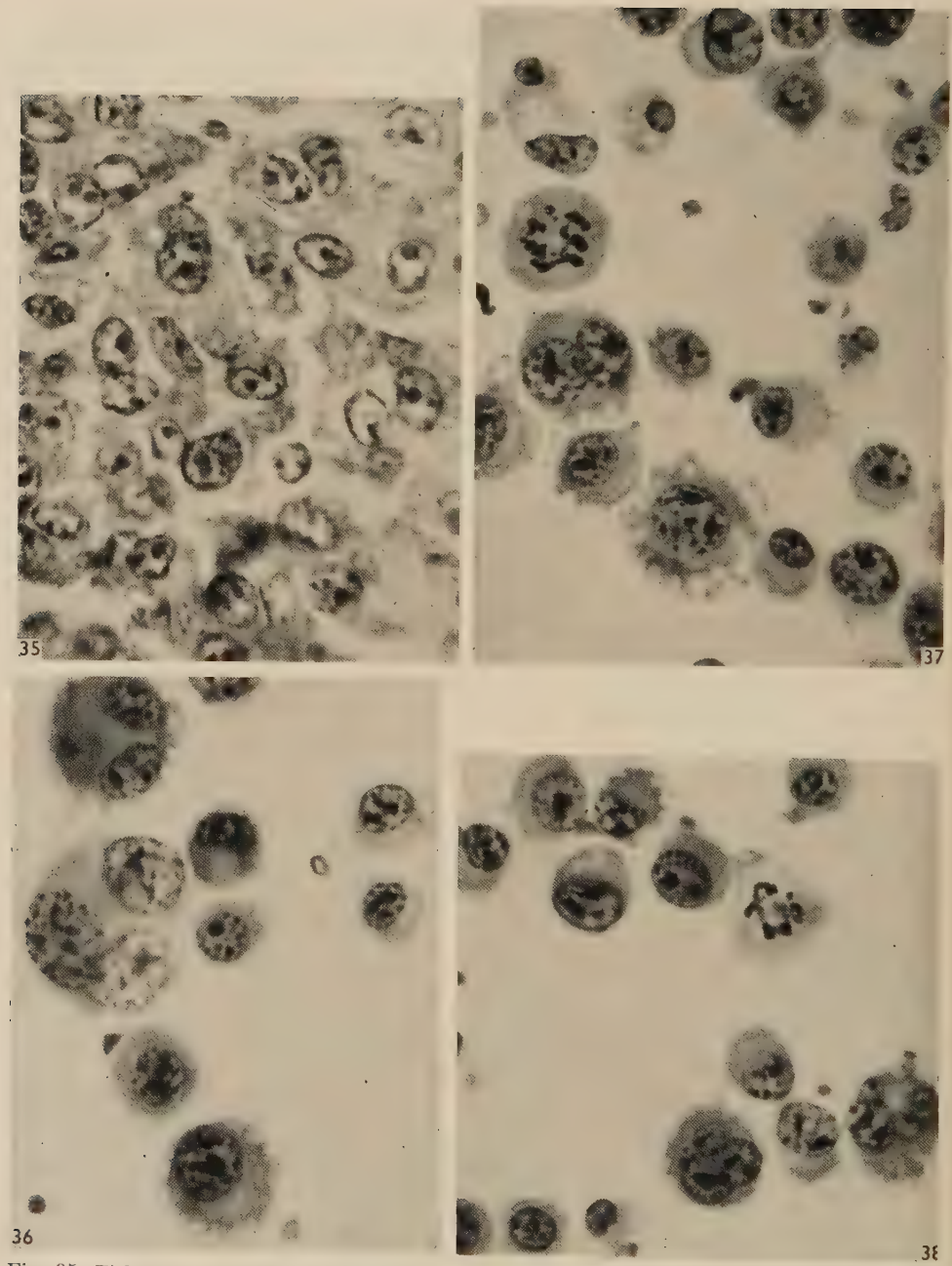
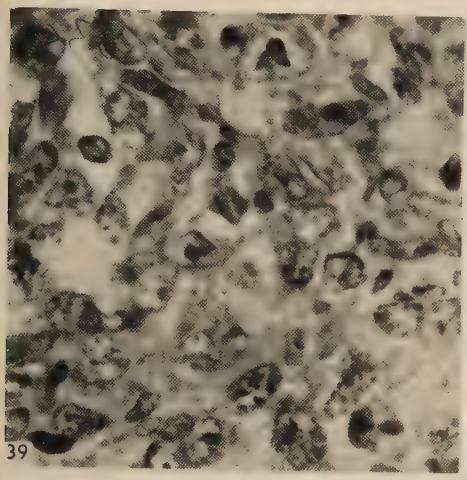
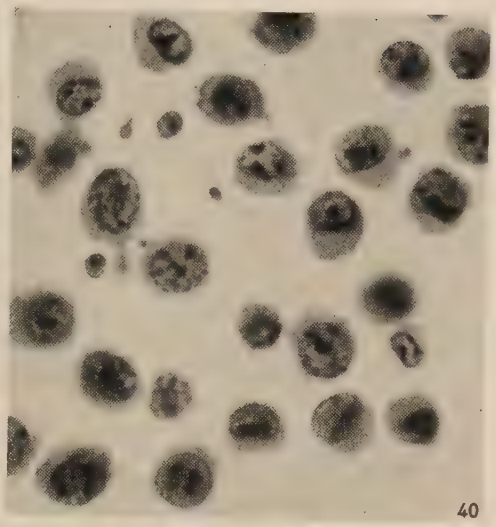


Fig. 35. Field of solid MC1M sarcoma.
Figs. 36–38. Free T cells from MC1M peritoneal exudates after 14–15 FTGs. Note hyperchromatic T cells with large nucleoli, presence of multinucleated cells, mitotic figures and great variations in cell size. Note histiocytes and mesothelial cells in Figs. 37–38.





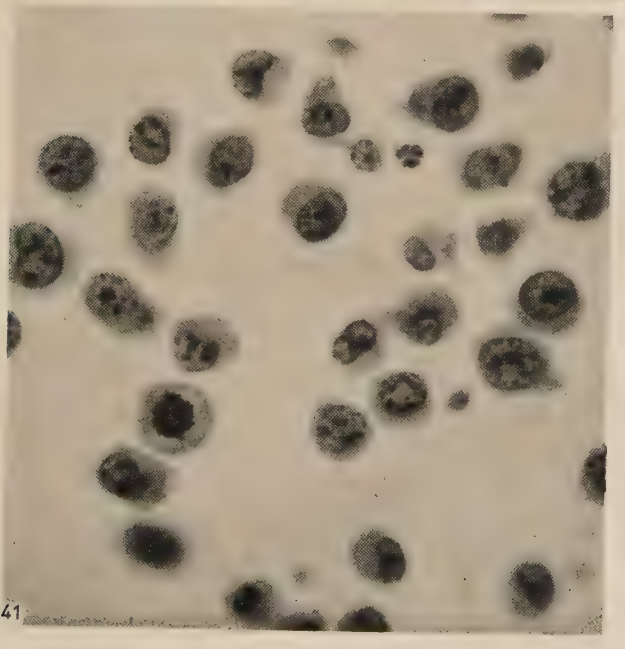
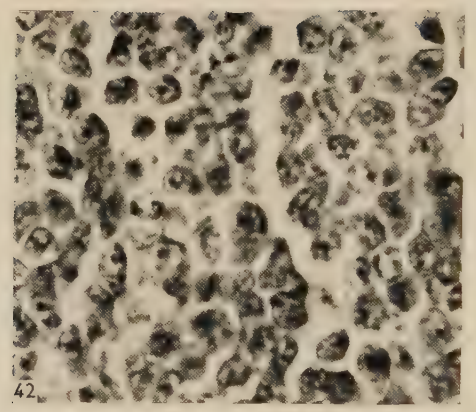
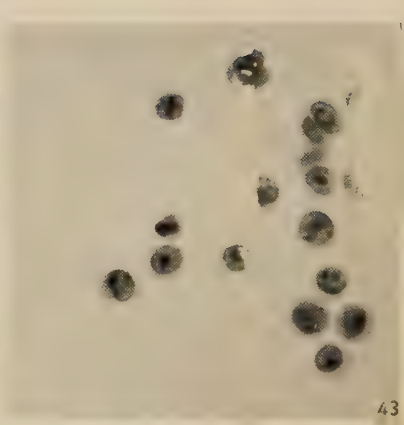
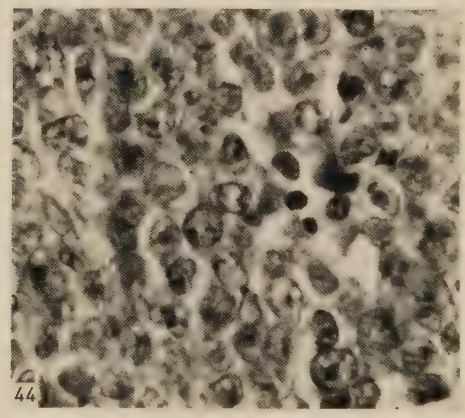
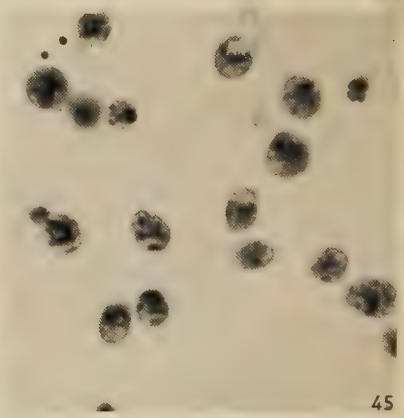


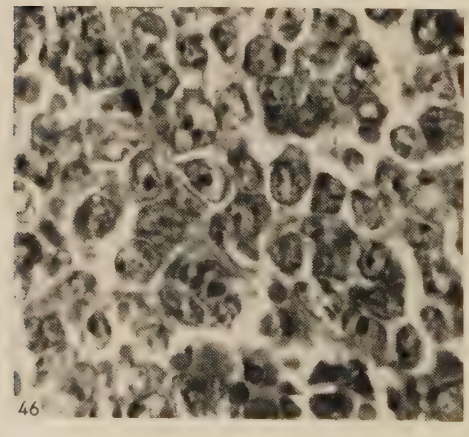
Fig. 39. Field of solid sarcoma 37.
Figs. 40–41. Free T cells from Sarcoma 37 peritoneal exudate. Note smaller sized T cells, not too great variations in size, and occasional large nucleoli. Many histiocytes and mesothelial cells are present, especially in Fig. 41.


42

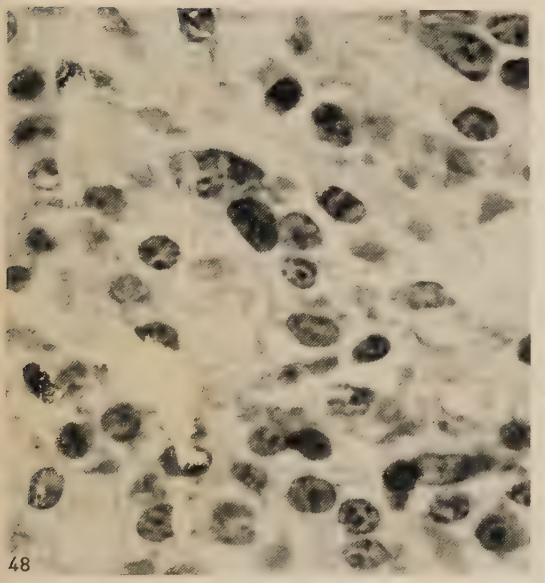

43


44


45


46

47



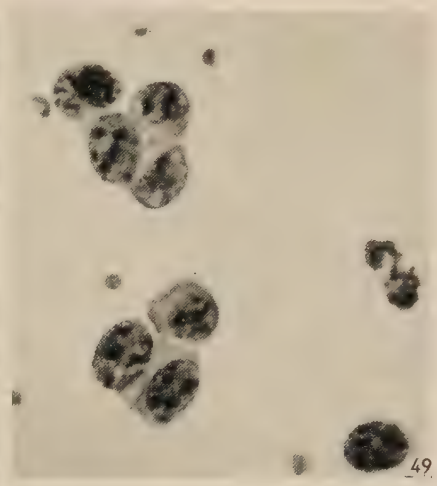


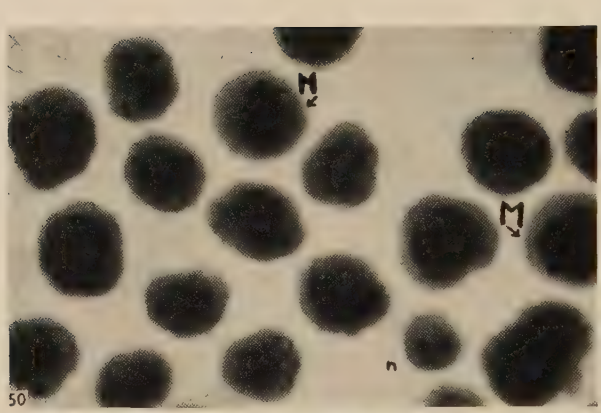
Fig. 48. Field of solid C 1300 tumor.
Fig. 49. Small T cell complexes of FTG 6 of tumor C 1300.

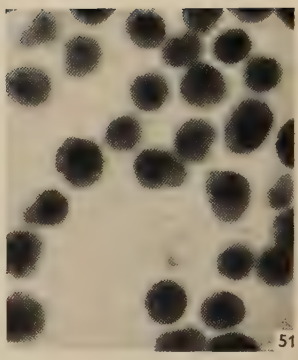
made their study comparatively easy. Less easy was the differential counting in the samples of the earlier generations with low frequencies of T cells, but careful studies of the corresponding fully developed ascites tumor on one hand and of the non-neoplastic cell types, occurring in neoplastic exudates, on the other hand, made such a differentiation possible in most cases. In the case of the lymphomas, diagnosis of neoplastic cells was more difficult, as stressed above.

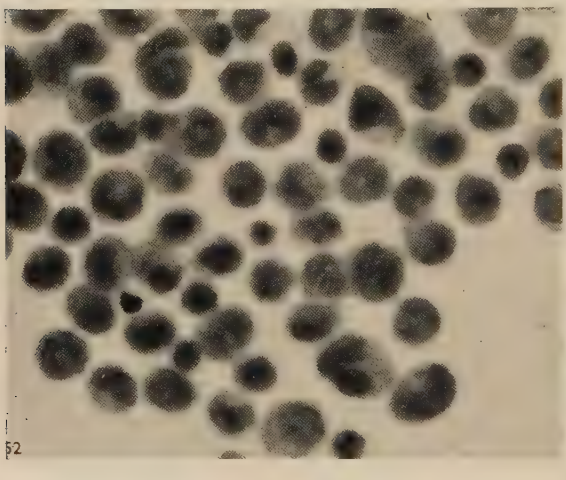
Two findings may be mentioned which seem to confirm the correctness of T cell differential countings. One is the correlation which was found between the number of cells diagnosed as T cells in the inoculum and the survival times of the animals. There was a tremendous variability between the absolute and relative cell content of different exudates with different

---

Fig. 42. Field of 6C3HED solid tumor.
Fig. 43. Ascitic fluid of FTG 12, lymphoma 6C3HED. Note small sized lymphoblast-like cells, in some cases with large nucleoli. One pyknosis.
Fig. 44. Field of solid dba lymphoma.
Fig. 45. Ascitic fluid of FTG 14, dba lymphoma. Note cells similar to those in Fig.43. One pyknosis.
Fig. 46. Field of solid Patterson lymphoma.
Fig. 47. Ascitic fluid of FTG 3, Patterson lymphoma.







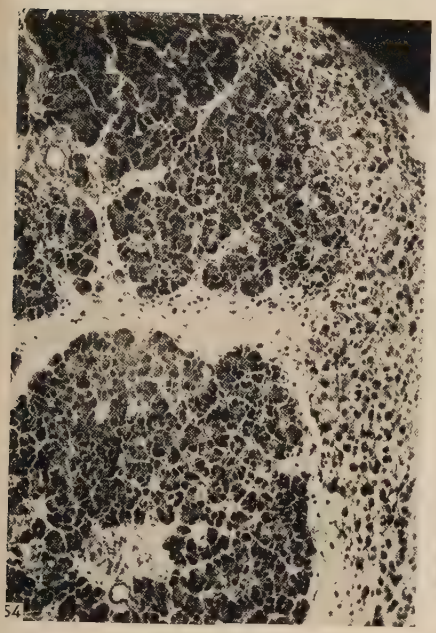
Figs. 50–53. Ultraviolet photomicrographs at 257 mμ. Illumination of the object according to Köhlers principle. Obj. ap. 0.85, cond. ap. 0.6. Magnification 720 x. Figs. 50–52 were exposed, developed and printed under strictly identical conditions. In Fig. 53, the conditions were different.

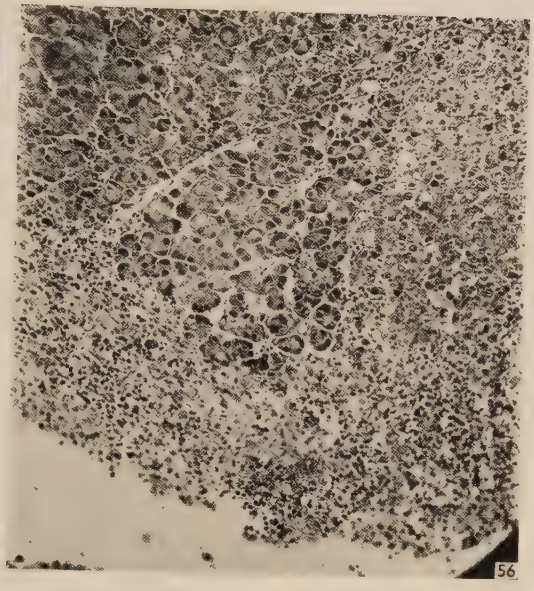
Fig. 50. Ehrlich ascites T cells. The cells were focused in the visible and exposed to U.–V. light only during the 10 seconds of exposure. At *n*, a non-tumorous exudate cell may be noted. Note large cell size and high absorption in T cells. At *M*, two mitotic figures.

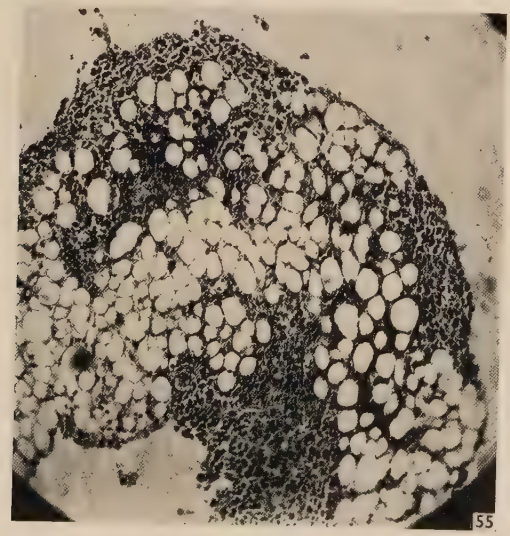
Fig. 51. Ascites cells of dba lymphoma, prepared similarly to cells in Fig. 50.

Fig. 52. Cells of normal peritoneal fluid from a C57 leaden mouse. Conditions the same as in Fig. 50.

Fig. 53. Nuclei isolated in citric acid of the Ehrlich ascites tumor to show the nuclear details which are in Fig. 50 covered by cytoplasmic absorption.







igs. 54–55. Beginning tumorous infiltration of the pancreas and mesenteric fat 4 days after the inoculation of 20 million Ehrlich ascites T cells. Magnification 90 ×.

ig. 56. Pronounced infiltration of the pancreas 8 days after inoculation of the same number of cells as in Figs. 54–55. Magnification 90 ×.

tumors in different generations. In spite of this, survival times of the animals inoculated with exudates containing different percentages of T cells seem to depend upon the number of what was diagnosed as T cells. This is illustrated by Figs. 1, 2, and 3, where there was an inverse relationship to be found between the number of T cells and survival time. Parallel to an increase in T cells on subsequent generations there was a decrease in survival times. On the other hand, in the case of the lymphomas, no definite changes occurred in the frequency of the cells counted as neoplastic and survival times were similarly constant (Fig. 6). The other fact supporting correctness of T cell diagnosis is the correlation between frequency of T cells and average DNA content per cell in the case of the tumors where a higher than normal DNA value per cell could be demonstrated. In all these tumors the DNA value increased with an increasing frequency of T cells in the analyzed sample. For the Ehrlich ascites tumor this is shown in Fig. 10. No such increase could be observed with tumors having the normal DNA value per cell, thus mainly the lymphomas examined. No such correlation could be expected to be found if the cells counted as T cells would to any considerable degree be mesothelial elements, but the values would be distributed more or less at random.

II. *Behaviour of the different tumors tested during the serial fluid transplant generations*

On the basis of the results presented the tumors tested may be divided into the following groups:

a) The tumors hepatoma 98/15 (No. 8), MC1N (No. 16), sarcoma 180 (No. 19) and Harding Passey melanoma (No. 27) gave only solid tumors and no exudate in all animals inoculated. These findings are only tentative, however, as on first trial the same was found with Sarcoma 37 (No. 18) and MC1E (No. 14). These tumors have been tried a second time, using larger inocula, and this time exudate formation was the result in a number of animals. Thus, the failure of tumors of this group to produce appreciable amounts of exudate may perhaps be overcome by further trials.

b) Tumors SPdba (No. 12), ICR (No. 3), melanoma S 91 (No. 28) and Wagner osteogenic sarcoma (No. 20) have also evoked exudates in a number of inoculated animals, besides solid tumor formation. These exudates, however, failed to transmit the tumors further. In no cases were there any T cells detected in the exudates. This failure of transmission, again, may not be the general rule.

c) All other tumors tested gave rise to exudate formation in a certain number of the animals inoculated, besides solid tumors or infiltrations of the peritoneal cavity.

Two very different and distinct types of behaviour can be distinguished in this group, however. On the one hand, there were tumors which upon their first intraperitoneal inoculation, immediately gave rise to exudates with a high frequency of homogeneously suspended free neoplastic cells. To this group belong all 4 lymphomas tested (Nos. 22, 23, 24, 25). The exact numerical evaluation of the malignant lymphoma cells is open to the objections mentioned on page 529, but a comparatively high frequency of lymphoma cells is still obvious as the exudates show a surprisingly homogeneous picture. On later transplants, the lymphomas showed a very constant behaviour, always presenting an exudate with high frequency of lymphoma cells, and with very constant survival times. None of the other tumors tested gave a T cell frequency higher than about 10 per cent in the exudates produced after the first intraperitoneal inoculation, with the only exception of Sarcoma 37, where the figure was 40 per cent. Sarcoma 37 showed a very irregular and capricious behaviour in later FTGs, but it can be stated in general that, similarly to the lymphomas, T cell frequency was independent of FTG number, though showing a much greater variability in the case of Sarcoma 37 than in the case of the lymphomas. The 4 lymphomas tested seem indeed to present a special group on the basis of the characteristics mentioned on page 540, to which now their uniform and low DNA content per cell may be added.

The other part of the tumors showed a different behaviour. The exudates which developed after the first intraperitoneal inoculation of the solid were either so poor in T cells that their presence could not be detected in the fields examined (but which presence still follows from the fact that the exudates transmitted the tumor) or their frequency was below 10 per cent. All the exudates in this group have been submitted to further serial intraperitoneal transplantations. During these transplantations (FTGs) 5 of the tumors tested showed a definite trend towards an increasing T cell frequency after a certain number of FTGs. The tumors, for which this could be established without doubt, were the TA3 (No. 1), 15091a (No. 2) and Krebs 2 (No. 5, ref. 24) carcinomas and the MC1A (No. 13) and MC1M (No. 15) sarcomas. All of these have passed at least 12 FTGs and the most conspicuous increase generally occurred around FTG 6–10, with the exception of Krebs 2, where it occurred between 2 and 4. The resulting fluids differed from each other not only by morphology of their T cells, but also

by other different characteristics. TA3 had an obstinate tendency to form cell complexes. Two generations after having started the FTGs with this tumor, Dr. T. S. Hauschka also established another subline of exudate-transfers, starting out with a solid subcutaneous TA3 tumor. His subline has presented the same picture: occurrence of a large number of T cell complexes in the exudates after a number of FTGs which could not be overcome by differential centrifugation of the exudate prior to transfer. This seemed to be a constant and characteristic feature of this tumor. Carcinoma 15091a gave exudates that were consistently and in all cases very bloody while the presence of gross blood was very variable with most other tumors. Krebs 2 reached the highest frequency of T cells, and showed a picture very similar to the Ehrlich ascites tumor. Both the sarcomas, MC1A and MC1M, reached concentrations of only 20–25 and 50–60 per cent of T cells, respectively, which were morphologically rather similar in both cases. A tendency for infiltration of the hepatic porta and consequent icterus was present in both tumors.

With all five tumors, there was a conspicuous decrease in the amount of macro- and microscopical infiltration of the peritoneal cavity during subsequent FTGs. This decrease was parallel to the increase of T cell frequency in the fluid and to a decrease of survival time. This phenomenon seems to be analogous to the previous findings with the Ehrlich and Krebs 2 ascites tumors (24) where it was found that the amount of solid tumors and infiltrations depends on the number of cells inoculated and on survival time, respectively, and increases with increasing survival times. It is supposed to be due to the fact that the injected T cells from exudates, which have passed a certain number of FTGs, or otherwise from established ascites tumors, if they are injected in a high enough number, start multiplying first freely in the peritoneal fluid, and solid infiltration of the peritoneal structures is a secondary phenomenon which depends upon how long the primary multiplication in the fluid permits the animal to survive. That such active multiplication in the fluid really occurs, is also evident from the experiment reported on page 553, where it has been shown for the Ehrlich ascites tumor, that after four days about 5 times as many tumor cells can be recovered from the ascitic fluid as have been injected, while solid infiltration is still hard to demonstrate, and, where found, seems to be just starting.

Injection of 20×10^6 Ehrlich ascites tumor cells which lead to death in 50 per cent of the animals in about 6 days, produced at this time 5–10 ml of ascitic fluid with a cell concentration of 130×10^6 on an average. Solid

infiltration at this time is still of very little extent and macroscopically not visible. On the other hand, after injection of very low cell numbers the animals live much longer and develop many solid infiltrates and tumors. Then, however, the ascitic fluids themselves are often less rich in T cells and contain more inflammatory cells. These observations, together with the fact that, after injection of high cell numbers, viable and well preserved T cells with numerous mitotic figures are to be found during the whole course of development of the Ehrlich ascites tumor and much earlier than any organized growth on the peritoneum could have occurred, all weigh against the possibility that the free T cells in the exudates in question are only desquamated cells from solid tumors on the peritoneum. Another proof for the primary multiplication of T cells in the peritoneal fluid has been presented in the case of Sarcoma 37 by Goldie and Felix (13). (Multiplication of T cells during rapid serial transfers, before vascularization of solid implants can start.)

The rest of the tumors tested have also evoked development of hemorrhagic exudates after the first intraperitoneal inoculation with no or only few detectable T cells, but, so far, no certain tendency towards increasing T cell frequencies could be observed in the subsequent FTGs. This may be due to the fact that not enough FTGs have been performed as yet. Thus, the three spontaneous mammary adenocarcinomas tested, SPAD (No. 9), SPAH (No. 10) and SPC3H (No. 11), respectively, had a much slower growth rate than the transplanted carcinomas used, a well known phenomenon. These tumors have passed only 3 FTGs. The tumors EO 771 (No. 4), C 954 (No. 7), T 241 (No. 17), MC1E (No. 14) and C 1300 (No. 26) have passed 4, 6, 8, 8, and 7 FTGs, respectively. A tendency towards increasing T cell frequencies is noticeable in the cases of C 954, MC1E, and C 1300 already with this small number of FTGs. However, further work is necessary for firmly establishing whether this is a definite trend. A disturbing factor in the case of C 1300 was that in all FTGs about 50 per cent of the animals developed only solid tumors with no exudate formation. None of the other tumors tested showed this behaviour. On the contrary, if the exudate transmitted the tumor, an increasingly smaller percentage, if any, of the animals in following FTGs showed solid tumor without exudate formation. This was evident already in the very early generations.

In order to try to explain the gradual transformation of the Krebs 2 solid into ascites tumor, we have previously (24) advanced the hypothesis of a possible *selection* of the cells or cell types, capable of survival and multi-

plication in the peritoneal fluid, during the subsequent FTGs by virtue of the fact that it is always the exudate that is transmitted. On the basis of the present experiments, this hypothesis can be maintained and further developed when considering the carcinomas and sarcomas tested. If there is an exudate present accompanying solid tumors of the peritoneal cavity, it is a matter of chance if and what T cells get into that exudate. By transplanting the exudate, the cells capable of living in the fluid will be the transmittors of the tumor. In the early generations, their relative frequency is so low that it escapes detection, or, if some T cells are detected, the establishment of their exact absolute and relative number will be very time-consuming if not impossible. However, after a certain critical number of FTGs,—different for different tumors—their frequency will increase, possibly due to further selection, reaching an easily countable range and up to a certain maximum value, where it stays rather constant.

An alternative possibility is that a gradual adaptation of the cells is taking place to the proliferation in the fluid medium of the exudate. A selection is perhaps made more probable by some preliminary experiments with the Krebs 2 tumor, where it was found that after the ascites form has been developed, subcutaneous inoculation of the ascitic fluids produced solid tumors, which, after having grown as solid subcutaneous tumors over 3 generations, immediately again gave rise to ascites tumor upon reinjection into the peritoneal cavity. The number of solid generations may however be too small to decide this question and further work is in progress.

It cannot be decided on the basis of the present experiments whether all tumors can be transformed into ascites tumors or not. This seems to depend on at least the following three factors: whether the tumors produce peritoneal exudates, into which tumor cells may get in. Second, whether there are any tumor cells able to proliferate in the fluid. Thirdly, whether the cells which are capable to live in the fluid do or do not have an inherent tendency to build cell complexes, as was the case with carcinoma TA3. Our "negative" tumors may have been accidentally so as illustrated by the history of MC1E, SPC3H and Sc37, and those, where the T cell frequency did not increase sufficiently, may require a higher number of fluid transplant generations. It has been demonstrated by the present experiments that the phenomenon of growth as an "ascites tumor" is not restricted to one morphological type of neoplasm.

As pointed out above, the lymphomas tested behave in an entirely different way, as they reach the stage of high T cell frequency in the fluid already after the first inoculation of the solid tumor. All lymphomas tested

behaved in this same way, and Sarcoma 37 was somewhat similar. In the case of the lymphomas the tentative explanation may be advanced, that this behaviour is due to the fact that the neoplastic cells originate from cells which are able to live in a fluid medium, both normally and after neoplastic transformation. Thus no further selection would be necessary for their immediate multiplication in the peritoneal fluid. No explanation can be attempted in the case of Sarcoma 37, on the basis of our experiments. However, Goldie and Felix (13) found that after a number of transplant generations with Sarcoma 37, the inoculum dose had to be reduced in order to get the same survival time. Before reaching this stage, they always selected the best fluid, i.e. the fluid showing the highest number of sarcoma cells and their mitoses. In our experiments no such selection was made and, possibly because of this difference, no dependence of survival times on number of transfer generation was found. However, the findings of Goldie and Felix point towards the necessity of selection of sarcoma cells in order to enable them to reach a better proliferation in the fluid, and thus classify Sarcoma 37 together with the other sarcomas and carcinomas tested, rather than with the lymphomas which showed no difference whatsoever on different FTGs.

It is of interest to point out that Goldie and Felix chose for their experiments just two tumors (Sc 37 and dba lymphoma) which produced exudates with many T cells already on the first transfer. It would not have been possible to carry out immediately their comparisons between the behaviour of "tumor mash cells" and free peritoneal neoplastic cells, should they have chosen any of the tumors which reach the concentration of T cells in the peritoneal fluid necessary to this type of work only after a number of FTGs.

As to the morphological appearance of T cells in the case of different tumors, rather different types were to be found. Indistinguishable from each other are the cells from the Ehrlich and Krebs 2 ascites tumors (Figs. 27, 28, 30), and the cells from the MC1A and MC1M sarcomas (Figs. 32–34, 36–38). This applies also to the 4 lymphomas (Figs. 43, 45, 47). The other tumors which produced exudates with high T cell frequency, have their own very characteristic cell types. This is shown in Figs. 19–21, 23–25, 40–41, 49. It is interesting to note that there are no great differences existing between the mitotic indices of different tumors around median survival time and between medial survival times themselves in the case of the different tumors where a higher frequency of T cells in the fluid could be attained. This may be due to the identical conditions under which

further that such an increase over the normal range of DNA/cell is not a phenomenon which is necessarily correlated with neoplasia.

One factor to be mentioned is the very different cell size of the lymphoma cells on the one hand and the carcinoma and mouse sarcoma cells from tumors where nucleic acid determinations could be carried out, on the other hand. The former were much smaller and variations in size were not too pronounced. All the carcinomas and mouse sarcomas in question, though showing great variations in cell and nuclear size, still in general consisted of cells that were much larger and had more voluminous nuclei than the lymphoma cells. It was just this factor which made the differential counting of them comparatively easy while differential counting of lymphoma cells was more uncertain, as the cells were in the size-range of small mesothelial cells and histiocytes, as pointed out before. The Yoshida rat sarcoma had an intermediate cell size, and also an intermediate DNA value. Thus there appears to be a rough correlation between cellular or nuclear size and mean DNA/cell values in the tumors examined, an additional indication that polyploidy may account for the differences found.

SUMMARY

The advantages presented by the use of the comparatively homogeneous free tumor cell suspension of the Ehrlich ascites tumor for quantitative studies on the growth and chemical composition of tumor cells and for chemotherapeutic experiments have led to the present investigation of a representative array of 26 histologically and genetically different mouse tumors which were tested for their capacity of reaching a condition similar to the Ehrlich ascites tumor. This has been found to be possible in a number of cases. Dissociated solid tumor masses were intraperitoneally inoculated into the corresponding mouse strain. Tumor growth on the peritoneum, accompanied by exudate formation, resulted in 22 out of 26 cases. The resulting exudates, which, in the case of all carcinomas and most sarcomas tested, contained very few tumor cells, transmitted the tumors upon further intraperitoneal injection in 18 instances. The tumors which developed were now regularly provoking exudate formation, and on subsequent generations the exudates have always been transplanted intraperitoneally. In the case of all carcinomas and in 2 out of 3 sarcomas tested which have passed over 10 such "fluid transplant generations" (FTG), a gradual increase in the frequency of tumor cells in the ascitic fluids (examined around median survival time) was occurring after a certain

number of FTGs and reached high values in some cases. At the same time, there was an apparent decrease in survival times of the inoculated animals and also a decrease in the extent of solid tumors and infiltrations in the peritoneal cavity. A different course of events could be observed for all 4 lymphomas examined. Here a high frequency of free neoplastic cells in the ascitic fluid was reached immediately after the first i.p. inoculation, which was maintained on later FTGs. It is supposed that in the case of the former tumors, a selection of the particular tumor cells which are able to live in the fluid medium of the exudate, has resulted from the serial transfers of the ascitic fluids. The lymphomas did not require such a selection, perhaps because of the capability of the cells to live in a fluid medium under both normal and neoplastic conditions.

Ascitic fluids collected around median survival time in different tumors were studied as to the morphological appearance and mitotic index of their tumor cells. The possibilities for the differential counting of tumor from non tumorous exudate cells in the case of different tumors were investigated and are discussed. On the basis of the relationships between solid growth on the peritoneum and concentration of free tumor cells in the ascitic fluid the hypothesis is advanced that under certain conditions the intraperitoneally injected tumor cells start multiplying in the peritoneal fluid and solid infiltrative growth on the peritoneum occurs only later.

Nucleic acid determinations on the ascitic fluids having high concentrations of tumor cells show that the mean desoxypentosenucleic acid (DNA) content per cell is different in different tumors. In some instances it is identical with the values established for normal diploid mouse cells and is elevated in others. The average DNA per cell values in different tumors do not show any definite correlation with mitotic index, but show a rough parallelism with cell size. PNA/DNA ratios are higher in tumors with elevated DNA/cell values than in those with "normal" values.

ACKNOWLEDGEMENTS

This work was carried out on a grant from Knut and Alice Wallenberg's Foundation. My sincere thanks are due to Prof. T. Caspersson, Dr. T. S. Hauschka, Dr. J. Schultz and Dr. C. Leuchtenberger for their interest in these investigations and their most valuable advice and criticism. I wish to thank Drs. Dalton, Diller, Fekete, Hankwitz, Hogeboom, Krebs, Miller, Sugiura, and Yoshida for the tumors obtained and to Miss Elin Klein for valuable technical assistance. I also wish to express my gratitude to the entire staffs of the Institute for Cell Research in Stockholm and the Institute for Cancer Research in Philadelphia for all their kind help during the course of this work.

Note added in proof.

Since this paper has been completed, Dr. H. Goldie has communicated to the author that he has been able to carry Sarcoma 180, two carcinomas in C3H and CAF1 mice, resp., and a melanoma in dba mice through serial intraperitoneal transfers, using tumor-cell containing peritoneal exudates.

REFERENCES

1. Boivin, A., Vendrely, R., and Vendrely, C., *Compt. rend.*, **226**, 1061 (1948).
2. Brodersen, H., *Strahlentherapie*, **73**, 196 (1943).
3. Caspersson, T., and Santesson, L., *Acta Radiol. Suppl.*, **46** (1942).
4. Cloudman, A. M., *Am. J. Cancer*, **16**, 568 (1932).
5. —— *Science*, **93**, 380 (1941).
6. Cunningham, L., Griffin, A. C., and Luck, J. M., *J. Gen. Physiol.*, **34**, 59 (1950).
7. Davidson, J. N., and Leslie, I., *Nature*, **165**, 49 (1950).
8. —— *Cancer Research*, **10**, 587 (1950).
9. Davidson, J. N., Leslie, I., and White, J. C., *Biochem. J.*, **47**, Proc. xvi (1950).
10. Davidson, J. N., and McIndoe, W. M., unpublished results, quoted from ref. 8.
11. Edwards, J. E., Dalton, A. J., and Andervont, H. B., *J. Nat. Cancer Inst.*, **2**, 555 (1942).
12. Goldberg, L., Klein, E., and Klein, G., *Exptl. Cell Res.*, **1**, 543 (1950).
13. Goldie, H., and Felix, M. D., *Cancer Research*, **11**, 73 (1951).
14. Gorer, P. A., *Brit. J. Cancer*, **4**, 372 (1950).
15. Graham, G. S., *Am. J. Path.*, **9**, 701 (1933).
16. Harding, H. E., and Passey, R. D., *J. Path. Bact.*, **33**, 417 (1930).
17. Ivers, J. B., and Pomerat, C. M., *Tex. Rep. Biol. Med.*, **5**, 92 (1947).
18. Jones, F. S., and Rous, P., *J. Exptl. Med.*, **20**, 404 (1914).
19. Karp, H., *Z. Krebsforsch.*, **36**, 579 (1932).
20. Klein, E., and Klein, G., *Nature*, **166**, 832 (1950).
21. Klein, E., Kurnick, N. B., and Klein, G., *Exptl. Cell Res.*, **1**, 127 (1950).
22. Klein, G., *Cancer*, **3**, 1052 (1950).
23. —— *Exptl. Cell Res.*, **2**, 291 (1951).
24. Klein, G., and Klein, E., *Cancer Research*, **11**, 466 (1951).
25. Koprowska, I., and Koprowski, H., To be published.
26. Krebs, C., Thordarson, O., and Harbo, J., *Acta Radiol. Suppl.*, **44** (1942).
27. Lettré, H., *Z. Physiol. Chem.*, **268**, 59 (1941).
28. Leuchtenberger, C., Vendrely, R., and Vendrely, C., *Proc. Natl. Acad. Sci.*, **37**, 33 (1951).
29. Lits, F. J., Kirschbaum, M., and Strong, L. C., *Am. J. Cancer*, **34**, 196 (1938).
30. Loewenthal, H., and Jahn, G., *Z. Krebsforsch.*, **37**, 439 (1932).
31. Lücke, A., and Klebs, E., *Virchow's Arch. path Anat.*, **41**, 1 (1867).
32. MacDonald, E., and Mosher, W. A., *J. Franklin Inst.*, **251**, 183 (1951).
33. Métais, P., and Mandel, P., *Compt. Rend. Soc. Biol.*, **144**, 277 (1950).
34. Miller, G. L., Green, E. U., Kolb, J. J., and Miller, E. E., *Cancer Research*, **10**, 141 (1950).
35. Mirsky, A. E., and Ris, H., *Nature*, **163**, 666 (1949).
36. Pasteels, J., and Lison, L., *Compt. Rend.*, **230**, 780 (1950).
37. Peterman, M. L., and Schneider, R. H., *Cancer Research*, in press.
38. Phillips, S. K., and McDonald, J. R., *Am. J. Med. Sci.*, **216**, 121 (1948).
39. Price, J. M., and Laird, A. K., *Cancer Research*, **10**, 650 (1950).
40. Quensel, U., *Acta Med. Scand.*, **68**, 458 et *Suppl.* **23** (1928).
41. Saito, N., Ikeda, K., Shimizu, K., Mori, S., Nagai, J., and Uetake, K., *Gann*, **41**, 173 (1950).
42. Saphir, O., *Am. J. Clin. Path.*, **19**, 309 (1949).
43. Schmidt, G., Hecht, L., and Thannhauser, S. J., *J. Gen. Physiol.*, **31**, 203 (1947).

44. Schrek, R., *Am. J. Cancer*, **28**, 389 (1936).
45. Seeger, P. G., *Arch. Exper. Zellforsch.*, **22**, 356 (1938).
46. Sugiura, K., Hitchings, G. H., Cavalieri, L. F. and Stock, C. C., *Cancer Research*, **10**, 141 (1950).
47. Swift, H. H., *Physiol. Zoöl.*, **23**, 169 (1950).
48. Vincent Memorial Laboratory Staff, The Cytologic Diagnosis of Cancer, W. B. Saunders Comp., Philadelphia and London, (1950).
49. Wagner, A., Undersøgelser over cancerresistensen hos hvide mus. Pp. 17–19. Stiftsbogtrykkerie A/S, Aarhus, 1933.
50. Wihman, G., *Acta Med. Scand. Suppl.*, **130**, (1948).
51. Yoshida, T., *Gann*, **40**, 1 (1949).
52. Zadek, I., Handb. d. allg. Hämatol., Urban and Schwarzenberg, Berlin-Wien, p. 1376, 1932.

BRIEF NOTE

UNFERTILIZED RABBIT OVA IN P^{32}-AUTORADIOGRAPHY

ERIK ODEBLAD

Women's clinic, Karolinska Institutet, Sabbatsberg Hospital, Stockholm, Sweden

Received January 24, 1951

MATERIAL AND METHODS

10 adult female rabbits have been used. 2 were anoestric and untreated, 6 were anoestric and treated with human chorionic gonadotrophin (Pregnyl), as shown in table I, and 2 were pregnant. Two hours before death each rabbit received 1.2–6 m C of P^{32} intravenously as orthophosphate. Intravenous injection of barbiturate was used to kill the rabbits. Immediately after death the ovaries were fixed in a solution of 6 per cent formalin + $^1/_2$ per cent trichloracetic acid in distilled water, the first two hours at + 4° C, then at + 19° C for 22 hours. The ovaries were frozen-sectioned in 10 μ sections, mounted on glass slides, passed through 3 baths of neutral distilled water for $^1/_2$ hour, dried, and passed through 3 baths equal parts of ethanol and ether for $^1/_2$ hour. They were then dried and subjected to autoradiography. Gevaert no-screen X-ray film or Gevaert Dentus Rapid film was pressed directly against the section for 4–20 days in iron presses. After this exposure, development, with Gevaert G 209 A, was performed for 4 minutes at + 19° C. The resolution was approximately 25 μ. Control exposures with non-radioactive material gave no blackening of the film.

About 150 autoradiographs and the corresponding sections, stained with haemalun-eosin, were examined. Follicles having an apparent diameter of 500–1 000 μ, showing no signs of atresia, and containing centrally cut ova (with visible nucleolus), having an apparent diameter of 150–200 μ, were selected for study. It was possible to find 16 such subjects. The number of grains (N) was counted in oil immersion at 1 700 times of magnification, using eye-piece squared micrometer, each square corresponding to 37 μ^2. The number of grains per 37 μ^2 was counted in the central parts of the ova (N_o), in the adjacent granulosa cells (N_g) in the adjacent part of hollow space of the follicle (antrum folliculi) (N_a), if visible, and in the fog region (N_f). The ratios

$$\frac{N_o - N_f}{N_g - N_f} \text{ (I) and } \frac{N_a - N_f}{N_g - N_f} \text{ (II)}$$

were calculated and the statistical errors considered. Thus the exposure from the ovum was compared with that from the granulosa tissue (I) or the exposure from the antrum folliculi was compared with that from the granulosa tissue (I). The ex-

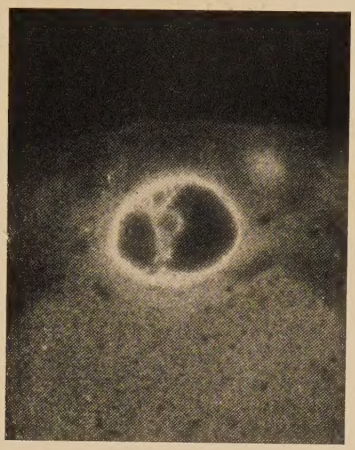


Fig. 1. Autoradiograph, enlarged 20 times, showing a follicle of a size of 900 μ, containing an ovum of about 150 μ size. The isotope distribution in the ovum, antrum and granulosa areas are clearly visible.

TABLE I

Rabbit nr	Hormonal treatment	Ratio I	Ratio II	Ratio I— Ratio II
1	Untreated, not pregnant	0.32±0.09	0.24±0.09	0.08±0.14
		0.35±0.10		
2	»	0.40±0.10		
3	Pregnyl[1] 5 days earlier	0.41±0.10		
		0.33±0.11		
4	»	0.51±0.08		
5	Pregnyl 15 days earlier	0.35±0.09		
		0.41±0.09	0.25±0.08	0.16±0.13
6	»	0.50±0.11		
7	Pregnyl 28 and 3 days earlier	0.41±0.08	0.23±0.07	0.17±0.11
		0.33±0.08		
8	»	0.42±0.12		
9	Untreated, pregnant 7 days	0.33±0.08	0.22±0.08	0.11±0.11
		0.39±0.09		
		0.40±0.09		
10	Untreated, pregnant 20 days	0.30±0.09	0.24±0.08	0.06±0.13
	Mean	0.39±0.04	0.24±0.05	0.15±0.06

[1] Each dosis of Pregnyl was 300 IU human chorionic gonadotrophin.

posure from the ovum was also compared with that of the antrum by subtraction of II from I.

In the region of exposure used the number of grains is approximately proportional to the amount of P^{32} emitting the radiation.

RESULTS

The results are shown in table I and examplified in fig. 1. The amount of P^{32} per unit tissue volume seems less within the ovum than within the granulosa tissue, but seems somewhat more than in the follicular fluid. No appreciable differences are seen between anoestric, hormone treated or pregnant rabbits.

DISCUSSION

The histologic treatment has with great probability dissolved much of the acid-soluble and lipid-soluble phosphorus. The remaining phosphorus in the section, therefore, must to a relatively great extent belong to nucleic acids and proteins.

Because of the dependence of exposure and resolution on the spatial distribution of isotope within the histologic section the results do not directly indicate the true amounts of P^{32} in the remaining fraction in the corresponding areas, but form a rough guide to the presence of P^{32} in different structures.

It is possible to criticize the calculation of the ratios because the number of granulosa cells varies from case to case. Due to similar degrees of follicular development, this variation is, however, slight.

This calculation has the avantage, that there is no appreciable dependence on the section thickness, dosage of isotope, length of exposure or time of development.

The findings are of interest with regard to the results of Lindberg (1, 2) and Villie, Lowens and Gordon (3) that unfertilized ova, contrary to fertilized ova, do not to any appreciable extent permit the penetration of labelled orthophosphate.

SUMMARY

By means of autoradiography, permitting a resolution of about 25 μ, it is indicated that acid- and lipid-insoluble P^{32} is present only in small amounts in unfertilized, nearly mature rabbit ova. No changes are indicated in treatment with human chorionic gonadotrophin or in pregnancy.

The author is indebted to the Pharmacia Co for having placed the gonadotrophic preparation Pregnyl at his disposal.

LITERATURE

1. Lindberg, O., *Arkiv. Kemi*, **26** B (1948).
2. —— *Exptl. Cell Res.*, **1**, 105 (1950).
3. Villie, C. A., Lowens, M., & Gordon, M., *J. Cellular Comp. Physiol.*, **33**, 93 (1949).